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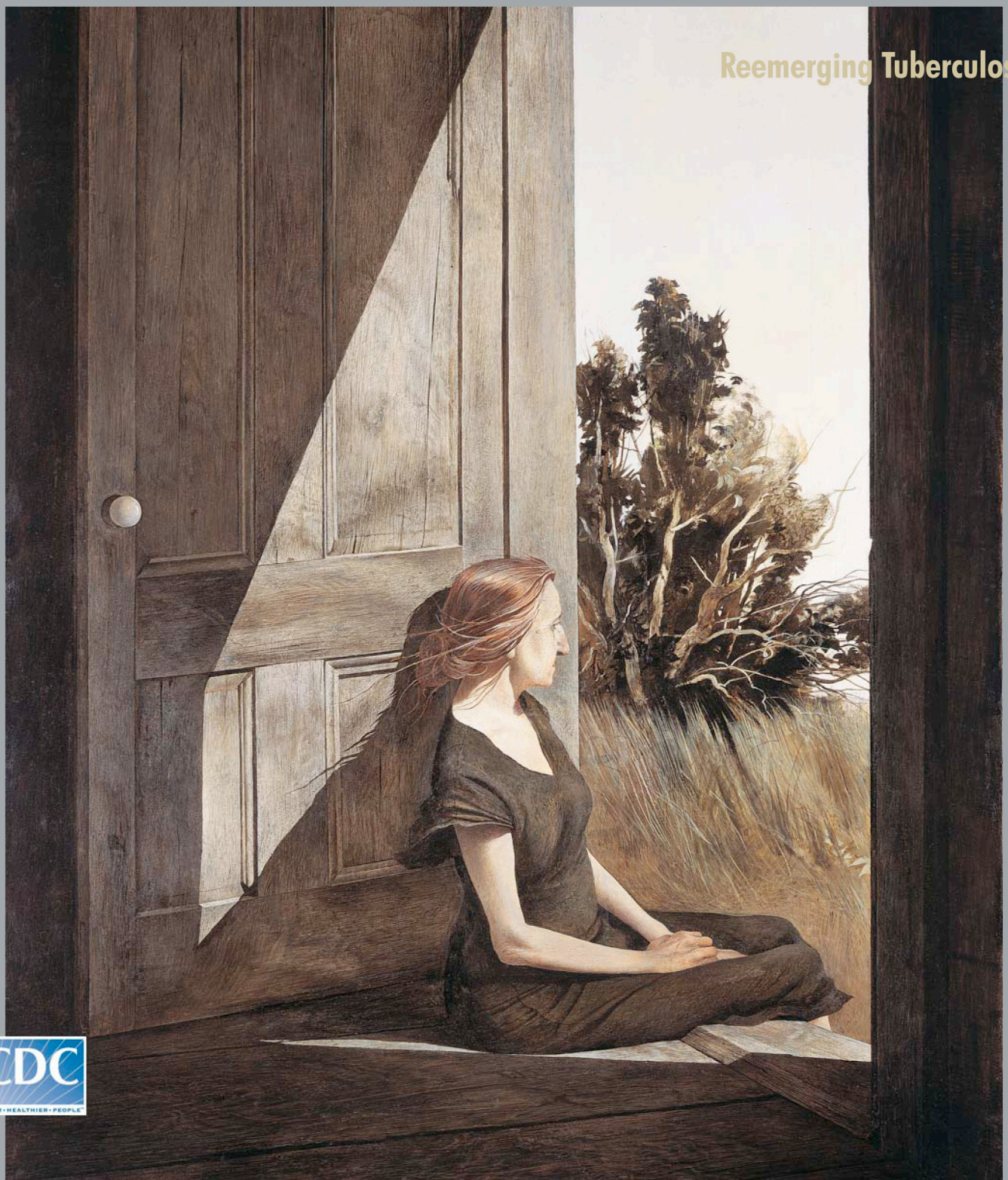

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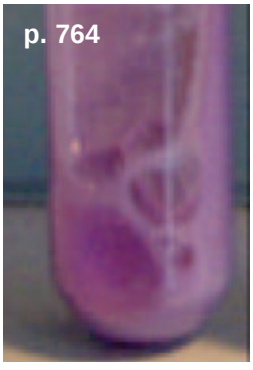
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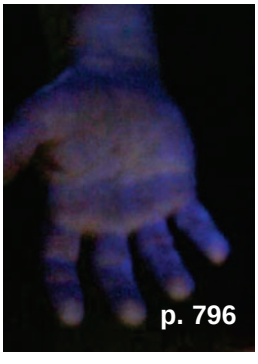
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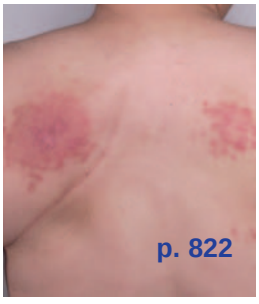

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Universal Genotyping in Tuberculosis Control Program, New York City, 2001–2003

Carla M. Clark,* Cynthia R. Driver,* Sonal S. Munsiff,*† Jeffrey R. Driscoll,‡ Barry N. Kreiswirth,§ Benyang Zhao,¶ Adeleh Ebrahimzadeh,¶ Max Salfinger,‡ Amy S. Piatek,* Jalaa' Abdelwahab,† and the New York City Molecular Epidemiology Working Group[1]

In 2001, New York City implemented genotyping to its tuberculosis (TB) control activities by using IS*6110* restriction fragment length polymorphism (RFLP) and spoligotyping to type isolates from culture-positive TB patients. Results are used to identify previously unknown links among genotypically clustered patients, unidentified sites of transmission, and potential false-positive cultures. From 2001 to 2003, spoligotype and IS*6110*-based RFLP results were obtained for 90.7% of eligible and 93.7% of submitted isolates. Fifty-nine (2.4%) of 2,437 patient isolates had false-positive culture results, and 205 genotype clusters were identified, with 2–81 cases per cluster. Cluster investigations yielded 57 additional links and 17 additional sites of transmission. Four additional TB cases were identified as a result of case finding initiated through cluster investigations. Length of unnecessary treatment decreased among patients with false-positive cultures.

Since the early 1990s, selective tuberculosis (TB) genotyping has been used in New York City for outbreak investigations, to identify isolates resistant to at least isoniazid and rifampin (multidrug-resistant TB), and in special studies. TB genotyping was essential to investigate and confirm transmission in a number of settings and to confirm or exclude laboratory contamination (*1–8*). A number of programs demonstrated the utility of universal genotyping, which influenced the development of this service in New York City (*9–16*). In 2001, the New York City Bureau of Tuberculosis Control began genotyping isolates for every new TB case with spoligotyping and IS*6110*-based restriction fragment length polymorphism (RFLP) to improve the efficiency of TB control. Two laboratories with extensive genotyping experience were selected through a competitive bidding process. Both were participating laboratories in the National Tuberculosis Genotyping and Surveillance Network and had performed genotyping for selected cases in New York City since the early 1990s (*6,17*).

The objectives of universal TB genotyping were to more rapidly and efficiently 1) determine the extent and dynamics of ongoing transmission to focus program interventions for specific areas and populations; 2) assess TB transmission in outbreaks to refine contact investigations; 3) identify nosocomial transmission not identified by conventional methods; and 4) identify false-positive cultures so that clinicians could be notified of diagnostic errors quickly and prevent unnecessary TB treatment. We describe the elements and activities required to develop and implement real-time universal genotyping in a large urban TB control program.

Identifying and Obtaining Isolates for Genotyping

Implementation of universal genotyping in New York City consisted, briefly, of 1) requiring submission of the initial positive isolate, reinforced by health code amendment (*18,19*); 2) advising all relevant laboratories and

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[1]In addition to the authors, members of the New York City Genotyping Working Group include Tracy Agerton, Sara Beatrice, Roseann Costarella, Rafael Fernandez, Dolores Gallagher, Karen Granville, Natalia Kurepina, Fabienne Laraque, Jiehui Li, Michelle Macaraig, Barun Mathema, Lucille Palumbo, Linda Parsons, Alex Ravikovitch, Harry Taber, Rachel Wiseman, and Genet Zickas.

providers of new requirements; 3) modifying laboratory submission forms; 4) establishing a specimen transport system; and 5) tracking and reviewing all submissions. In addition, protocols were developed for surveillance of genotype results and false-positive culture investigations, existing patient interview forms were modified, new databases were created, and program staff were informed through special trainings and newsletters. The New York City Department of Health and Mental Hygiene Institutional Review Board and the associate director for Science of the National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, reviewed the protocols, procedures, and modified data forms and determined that the genotyping service was not human subjects research since it would become a routine program activity.

Laboratory Procedures

An additional full-time staff person was hired by the Bureau of Tuberculosis Control to coordinate genotyping services. Spoligotyping and RFLP, respectively, were performed by the New York State Department of Health's Wadsworth Center in Albany, New York, and the Public Health Research Institute in Newark, New Jersey. This combination of genotyping methods is sensitive and specific for determining matching genotypes (*20–24*).

Isolates of *Mycobacterium tuberculosis* complex submitted to the public health laboratory from clinical laboratories were received on solid or liquid media and were stored at 4°C. Liquid media were prepared (10% glycerol in Dubos Davis broth with Tween and albumin), 1 mL of liquid culture was injected and incubated for 3 days at 37°C and checked visually for growth. Four freeze vials (1 for spoligotyping and 3 for archiving) and 1 Lowenstein-Jensen slant were injected. Mycobacteria in the tubes for spoligotyping were heat-killed at 80°C for 1 hour and mailed in biohazard containers to the Wadsworth Center. Once appropriate growth was obtained on the Lowenstein-Jensen slants, they were sent in a biohazard container to the Public Health Research Institute for IS*6110* RFLP analysis. Packages were mailed on a weekly or biweekly basis, depending on the number of isolates received. Spoligotype analysis was performed at the Wadsworth Center and given descriptive nomenclature according to a standard method (*25–27*). DNA analysis based on IS*6110* Southern blot hybridization was performed at the Public Health Research Institute with previously described methods (*28*,*29*). To ensure good communication, a working group of all partners in the genotyping service was formed. Regular telephone conferences were conducted to address issues such as quality and shipping of isolates and submission time for genotyping.

Creation of TB Genotyping Databases

Implementing universal genotyping also required developing a comprehensive database to monitor and manage information on specimen collection, shipment, and genotyping, as well as epidemiologic information gathered on each clustered patient. A relational database was created by New York City TB control staff in Microsoft Access (Microsoft Corporation, Redmond, WA, USA) that included 1) genotyping results for isolates identified after January 1, 2001; 2) specimen-tracking information such as date of receipt at the public health laboratory, shipment and reporting dates from each genotyping laboratory, and false-positive culture investigation results; 3) clustered patient information, such as location where each patient spent time during the potential infectious period, locations where TB could have been acquired in the 5 years before diagnosis, cluster characteristics, links between patients, and potential transmission sites; and 4) results of genotyping performed from 1990 to 2000 as part of the selective genotyping activities (*3*,*5*,*6*,*8*,*17*). Queries of the database were developed to identify cases with identical RFLP and spoligotype results for "real-time" cluster investigation and investigations of false-positive cultures. Quality assurance exercises to test reliability of results were developed and kept in the database. Queries are performed monthly to identify cases for which an isolate was not submitted to the public health laboratory. In such cases, Bureau of Tuberculosis Control staff sends reminder letters and makes phone calls to ensure that these isolates are received.

Application of Universal Genotyping Data

Investigation of False-positive Culture Results

A false-positive TB culture is defined as a positive TB culture that is not the result of culture-positive disease in a patient but instead may be due to 1) laboratory cross-contamination during specimen processing; 2) errors in collection or labeling, either on the patient ward or in the laboratory; or 3) contamination of clinical devices, for example, contamination of a bronchoscope during specimen collection. The primary goal of investigations of false-positive cultures is to discontinue unnecessary treatment in patients found to have false-positive TB cultures. Before universal genotyping, suspected false-positive cultures were investigated in 1 of 3 ways: 1) monthly review of patients with a single positive culture; 2) request from Bureau of Tuberculosis Control staff, including case managers, department of health physicians, and epidemiologists; and 3) requests by outside providers and laboratories to investigate cultures not consistent with the patient's clinical picture. With universal genotyping, an investigation can also be initiated when cases have matching

genotypes and have been processed within 2 working days of each other at the same facility. For these investigations, genotype information, specimen processing, and other information (e.g., patients hospitalized on the same floor) are reviewed, and suspected false-positive cultures are determined to be confirmed, unlikely, or inconclusive. Treating physicians and clinical staff in the program are notified of the outcome of investigations of false-positive cultures so patient evaluation can be evaluated further and a decision can be made on whether continued treatment is indicated.

Genotype Cluster Investigations

A cluster investigation aims to uncover epidemiologic links between members of a genotype cluster through systematic review of patient records and re-interviews, if needed. We consider real-time investigation of clusters to occur when the cluster investigation components (i.e., record review and re-interview) take place close to the time the most recent case in the cluster is identified. We defined a genotype cluster as ≥2 cases identified from 2001 to 2003 that had isolates with identical IS*6110*-based RFLP banding pattern and spoligotype, regardless of the number of IS*6110* copies. Patients with a definite epidemiologic link include those who have named each other as contacts, have a contact in common without naming each other as contacts, or have reported a common date range at the same location (e.g., residence, hospital, prison, workplace, single-room-occupancy hotel [any supervised publicly or privately operated facility designed to provide temporary living accommodations], or shelter). The common date range includes the potentially infectious period (i.e., 3 months before start of treatment) for at least 1 patient. Patients with a probable link have spent time at the same location (as above) during the same time frame, exclusive of the infectious period of the patients, without naming each other as contacts. Possible links exist among patients who have a similar social network or have spent time in the same area (no specific location), without naming each other as contacts.

When definite epidemiologic links are found among cluster members through the review of the TB case registry and patient records, information is recorded in the database on the nature of this relationship. If transmission at specific locations is shown, additional contacts are tested at these locations. If no such links exist, an epidemiologist reviews the cases and conducts in-depth patient re-interview to attempt to identify links and previously unidentified locations of transmission. A standard questionnaire is used to re-interview clustered patients. In addition, other registries such as the Department of Homeless Services are searched by cross-matching with the database each quarter to identify other possible exposure locations.

Performance Indicators

Performance indicators are used to evaluate procedures with respect to timely shipping of isolates and reporting of genotyping results and to assess the reliability of genotyping results. Submission time is calculated for clinical laboratories that process samples from New York City TB patients as the time between the date a positive culture is collected and the date the isolate is received at the public health laboratory. Submission time for genotyping is the time between the date the isolate is received at the public health laboratory and the date the isolate is sent for genotyping. Reporting time for the genotyping laboratories is the time between the date the specimen is received at the genotyping laboratory and the date the spoligotype or RFLP is reported to the Bureau of Tuberculosis Control.

The time to completion of investigations of false-positive cultures is defined as the time from specimen collection to investigation completion. The goal is to complete investigations within 90 days of collecting the first positive culture. Time to completion of a cluster investigation is calculated from the date a cluster is identified and an investigation is initiated until a decision is made regarding links between cases in the cluster; the goal is to complete these investigations within 21 days. Because clusters are dynamic, a new investigation is started when an additional case with that particular strain is identified.

Quality assurance exercises to assess the reliability of genotyping results are performed every 6 months. Ten percent of isolates genotyped in the previous 6 months are randomly selected by Bureau of Tuberculosis Control and sent for blinded retyping. Each laboratory repeats genotyping and sends the results to the bureau for comparison with previously reported results. Discrepant results are reviewed and discussed in the working group, and another isolate is requested from the initial processing laboratory to verify results.

Outcomes

The genotyping services process is summarized in the online Appendix Figure (available at http://www.cdc.gov/ncidid/EID/vol12no05/05-0446_appG.htm). The number of eligible isolates by year is shown in the Table. As of March 2004, isolates for 2,600 (96.8%) of 2,685 patients with a diagnosis of culture-positive TB from January 1, 2001, to December 31, 2003, were submitted. Of 85 patient isolates not submitted to the public health laboratory, 78.8% were processed at commercial laboratories, mostly outside of New York City. For patient isolates with incomplete genotyping (n = 163), RFLP could not be performed because of inadequate growth or overgrowth with other mycobacteria or fungi. Spoligotype and RFLP results were available for 2,437 (93.7%) of the 2,600 isolates submitted (90.7% of all culture-positive patients). The median

Table. Genotyping of isolates, New York City, 2001–2003*

Isolate characteristics	2001, no. (%)	2002, no. (%)	2003, no. (%)
Culture-positive	965 (100)	840 (100)	880 (100)
Culture received by PHL	928 (96.2)	808 (96.2)	864 (98.2)
Complete genotype (RFLP plus spoligotype)	883 (95.2)	772 (95.5)	782 (90.5)
Clustered genotypes	311 (36.3)†	262 (34.9)†	264 (34.2)†

*PHL, public health laboratory; RFLP, restriction fragment length polymorphism.
†Excludes false-positive cultures.

days from specimen collection to reporting of spoligotype decreased from 84 days in 2001 to 53 days in 2003, and the reporting time for RFLP patterns decreased from 127 days in 2001 to 78 days in 2003 (Figure). Fifty-nine (2.4%) isolates were false-positive cultures; 37% of investigations of these false-positive cultures were initiated through matching genotyping results or a spoligotype suggestive of contamination with a laboratory TB strain. Outside requests initiated 8.5% of investigations; 24.0% were initiated from the single positive culture list, and 30.5% by request from staff within the Bureau of Tuberculosis Control. The median time to complete investigations of false-positive cultures decreased from 178 days in 2001 to 85 days in 2003. In 2003, patients with a false-positive culture were treated unnecessarily for a median of 7 days (range 0–145). This median number of days is considerably lower than that seen before universal genotyping; in 1999, patients identified by retrospective surveillance (i.e., the single-positive culture list) as having false-positive cultures completed a median of 7 months of treatment.

Among 2,378 isolates with a complete genotype (true-positive cultures), 565 spoligotype patterns and 1,600 RFLP patterns were identified; 2,009 (84.5%) of 2,378 patient isolates clustered in 196 spoligotype clusters, and 1,002 (42.1%) of 2,378 patient isolates in 224 RFLP clusters. Eight hundred thirty-seven (35.2%) of the 2,378 isolates had RFLP and spoligotype patterns that matched ≥1 other isolate pattern; these isolate patterns were grouped into 205 genotype clusters ranging in size from 2 to 81 cases (mean 4 cases/cluster; median 2 cases/cluster). The percentage of clustered patient isolates remained stable during the 3-year period (χ^2 for trend p = 0.3652). While most patient isolates had 9–13 copies of IS*6110* (median 11 copies, range 1–23), strains with a lower copy number (<6 bands) were more likely to be clustered. From 2001 to 2003, two large outbreaks occurred that involved strains of 1 and 3 IS*6110* copies. After these strains were excluded, the percentage clustered remained higher for patterns with lower numbers of IS*6110*.

A total of 278 (33.2%) of 837 clustered cases had epidemiologic links identified; of these, 105 (37.8%) had links established through traditional contact investigations. Genotype cluster investigations established links for the remaining 62%: 15% of the links were definite, 11% probable, and 36% possible. For 66.4% of clustered cases (556 cases), no epidemiologic links were identified. Time to completion of cluster investigations decreased from a median of 176 days in 2001 to 37 days in 2003. The delay in completing investigations at the beginning of the project was mostly due to staff vacancies. Cluster investigations uncovered 57 additional links among cases with matching genotypes and 17 additional sites of transmission. Links established through genotype cluster investigations led to 4 expanded contact investigations in congregate settings (2 in homeless shelters, 1 in a single-room-occupancy hotel, and 1 in a local grocery store). These investigations identified additional infected contacts and 4 additional TB patients at a homeless shelter. These sites are now monitored closely for additional patient isolates with these genotypes. Transmission between TB patients was ruled out in ≥5 site investigations because the genotypes were unrelated, avoiding more extensive case-finding efforts that are needed once transmission is seen.

Four quality assurance exercises were performed from 2001 to 2003 on 216 isolates. The result was 94.4% concordance for spoligotyping and 93.5% for RFLP. Of retyped spoligotype patterns that did not exactly match the original patterns, 50% differed by ±1 spacer, 8% differed in multiple successive spacers, and 42% differed for other reasons. Among retyped RFLP patterns, 57% differed from the original patterns because of the existence or absence of ≥1 bands, 36% differed because of pattern shifts, and 7% differed for other reasons.

Discussion

We achieved real-time universal genotyping as part of routine TB control with capture and completion comparable to that seen by the National Tuberculosis Genotyping and Surveillance Network sites (*30*). High participation rates among clinical laboratories were essential to the completeness of genotyping. Timely submission of isolates from clinical laboratories and continuing decrease in submission time from the public health laboratory to the genotyping laboratories also facilitated efforts to achieve real-time investigation of false-positive cultures and clusters.

Implementation of TB genotyping in a large TB control program is complex. It requires TB control, epidemiology, and laboratory resources, and the costs are substantial. New York City contracts with genotyping laboratories carry an annual cost of nearly US $150,000 ($20,000 for

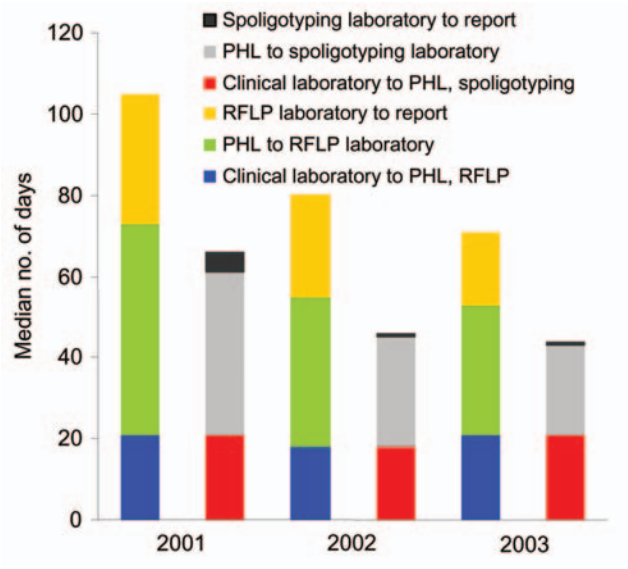

Figure. Median days for submission and turnaround time by laboratory, New York City, 2001–2003. PHL, public health laboratory; RFLP, restriction fragment length polymorphism.

spoligotyping and $125,000 for RFLP). In addition, 2 to 3 epidemiologists are allocated for database management and cluster investigation in New York City. Nonetheless, we have seen added value from universal genotyping. Additional sites of transmission were found on the basis of results of cluster investigations. Expanded investigations conducted at these sites identified additional patients and infected contacts who were subsequently treated for TB and latent TB infection. Genotyping information has also been useful by showing that TB cases clustered in place and time can have unrelated genotypes. For example, unrelated genotypes of ≥2 TB cases diagnosed in a setting with a high prevalence of TB infection may provide evidence that the cases did not occur as a result of transmission within that setting. Thus, more limited contact investigations of persons exposed to each of the patients can be performed instead of the more aggressive expanded contact investigation or case-finding activities that would be required if the isolates had matching genotypes. In addition, the efficiency of investigations of false-positive cultures increased as a result of universal genotyping, since a greater proportion of investigations initiated through genotyping matches yielded true false-positive culture results than investigations initiated through other methods. The amount of unnecessary treatment for these patients also decreased.

The higher rates of clustering seen in low copy-number isolates by RFLP alone support our decision to use 2 genotyping assays; this phenomenon has been reported previously (*11*). In addition, the rapid availability of spoligotype results allowed earlier initiation of investigations of both clusters and false-positive cultures than would have been possible with RFLP results alone. Particularly useful was close communication with the Wadsworth Center on interpretation of spoligotype matches for "rare" spoligotypes (seen less often than average for most spoligotypes in our database) and on prioritization of these isolates for investigation as clusters or false-positive cultures.

Since January 2004, mycobacterial interspersed repetitive unit and spoligotyping analyses are performed on all isolates as part of the Centers for Disease Control and Prevention's National Tuberculosis Genotyping Program. The availability of this additional assay will allow us to examine the extent to which MIRU further differentiates genotype clusters on the basis of RFLP and spoligotyping. MIRU may also reduce the time to obtain the genotype result and initiate a cluster investigation since it, like spoligotyping, requires few organisms and does not require live culture. Implementing the national genotyping service will also greatly reduce the financial costs for TB control jurisdictions interested in using genotyping to enhance their current program activities (*31*).

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Ms Clark is a research scientist and the genotyping services coordinator in the Epidemiology Office of the New York City Department of Health and Mental Hygiene, Bureau of Tuberculosis Control.

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Tuberculin Skin Testing in Children

Marina Reznik* and Philip O. Ozuah*

In 1996, the American Academy of Pediatrics (AAP) recommended targeted tuberculin skin testing (TST) of children while discouraging routine TST of children without risk factors for tuberculosis (TB). Recent studies have provided evidence in support of the targeted TST and recommendations that favor risk assessment over universal screening with TST. While evidence for targeted TB testing exists and benefits of screening programs are clear, administrative logistics could be a greater issue. The challenge for public health and school officials is to develop a screening program that avoids stigmatization of the at-risk group. Until then, pediatric healthcare providers will continue to have a key role in identifying children at risk for latent TB infection by using the AAP-endorsed risk-assessment questionnaire and should screen children with TST only when >1 risk factor is present.

In the 1960s and 1970s, when tuberculosis (TB) infection rates in the United States were high, universal screening for TB was required for all children (*1*). Between the 1980s and early 1990s, in response to a new increase in incidence of TB cases in the United States (*2–4*), the American Academy of Pediatrics (AAP) recommended annual tuberculin testing for high-risk children such as blacks, Hispanics, the socioeconomically disadvantaged, and children living in neighborhoods where the disease rate was higher than the national average (*5*). In 1996, the AAP's committee on infectious diseases (*6*) issued updated guidelines that called for targeted tuberculin skin testing (TST) of children and discouraged universal testing of children who lack risk factors. More recently, these recommendations were reiterated by a joint statement of the American Thoracic Society, the Centers for Disease Control and Prevention (CDC), and the Infectious Diseases Society of America (*7*). We review the rationale and evidence in support of targeted TST in children and discuss some of the logistic aspects of instituting targeted screening programs.

Who Is at Risk for TB Infection?

Targeted testing is intended to prevent progression of TB by identifying persons at risk for TB infection or disease who would benefit from treatment for latent TB infection (LTBI). Children at high risk for TB infection include contacts of persons with active TB; those who are foreign-born; those who travel to or have household visitors from a country with a high TB prevalence such as Mexico, the Philippines, Vietnam, India, and China (*8*); contacts with high-risk adults, including those who are homeless, incarcerated, infected with HIV, or intravenous drug users; and those with chronic conditions such as diabetes mellitus, renal failure, malnutrition, or other immunodeficiencies (*6,7*).

The rationale for targeted TB screening includes some of the following factors. The positive predictive value of any test, even one with high sensitivity and specificity, is extremely low in any population with low prevalence of the disease in question. Universal testing of such a population would lead to a low benefit-to-cost ratio. The sensitivity and specificity of TST are ≈90%, which results in a higher positive predictive value in high-prevalence populations (*9*). Among children with a 1% rate of TB infection, the positive predictive value is <10%. Thus, >90% of positive reactions are false positives (*10*). Since no test can distinguish false positives from true positives, all persons with positive TST results must be evaluated and treated. Falsely identifying TB in a child creates unnecessary cost for clinic visits, radiographs, treatment with isoniazid that has harmful side effects, family testing, and follow-up appointments. In addition, this false identification may cause anxiety as the physician and family try to determine the source of a nonexistent infection and create an ethical dilemma by labeling a child as infected with TB.

Benefit of Targeted TST in Children

Previous studies have shown a benefit of the targeted TST in children (*11–13*). In a study of 2,169 children who had mandatory TST because they resided in a high-prevalence community, Ozuah et al. (*11*) found a low rate (0.5%) of TST reactivity. These findings support the revised AAP guidelines recommending targeted TST of children at high risk for TB. Cost-effectiveness of school-based targeted

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TST compared with universal screening of children in the United States, as well as in other countries, showed that targeted screening of schoolchildren was more cost effective than mass screening (*12*,*13*).

Assessment of Risk Factors for LTBI in Children

Several recent studies have addressed the use of risk assessment to identify children who are likely to have reactive TST results (*14*–*18*). Although these studies assessed different populations, their findings were similar. Lobato et al. (*14*) conducted a case-control study in 953 children (<6 years of age) who had a TST read at public health clinics in California. Risk factors for a positive TST result (≥10 mm) among the study population included ≥1 week foreign travel to a country with a high prevalence of TB within the past 12 months (odds ratio [OR] 3.9, 95% confidence interval [CI] 1.9–7.9) or a household visitor from such a country (OR 2.4, 95% CI 1.0–5.5).

Saiman et al. (*15*) conducted a multicenter, prospective, matched, case-control study in children (1–5 years of age) in New York who underwent TST by primary care providers during routine healthcare visits. Of 288 persons, 96 were cases (defined as persons with a TST result ≥10 mm and a normal chest radiograph) and 192 were age- and clinic-matched controls (defined as subjects with a TST result = 0 mm). This study identified several risk factors for LTBI in children: contact with an adult with TB (risk ratio [RR] 61.6, p = 0.0004), foreign birth (RR 9.2, p<0.0001), foreign travel (RR 7.5, p = 0.0002), or a family member with LTBI (RR 15.7, p<0.0001).

In a similar study, Besser et al. (*16*) identified risk factors for LTBI in children (<6 years of age) in San Diego, California, who received a TST as part of routine well-child care. Fifty-one persons with a TST result ≥10 mm and normal chest radiograph and 72 age-matched controls participated in the study. In this population, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) immunization (OR 53, 95% CI 13–224), a TST within 12 months (OR 24, 95% CI 1.7–347), or a relative with a positive TST result (OR 4.9, 95% CI 1.4–16.5) were risk factors for LTBI.

Froehlich et al. (*17*) conducted a prospective observational study to determine if a risk-assessment questionnaire could predict a positive TST result in a population of 31,926 children (1–18 years of age) in California. This study found that BCG immunization (OR 2.3, 95% CI 1.7–3.1), foreign birth (OR 8.6, 95% CI 6.2–12.1), living outside the United States (OR 2.1, 95% CI 1.5–2.9), Asian (OR 2.3, 95% CI 1.6–3.3) or Hispanic (OR 1.6, 95% CI 1.1–2.3) ethnicity, or contact with a household member with LTBI or TB (OR 1.5, 95% CI 1.1–2.0) were independent predictors of LTBI.

Ozuah et al. (*18*) conducted a prospective criterion standard study of 2,920 children (1–18 years of age) in the south Bronx, New York, to determine the sensitivity, specificity, and predictive validity of the New York City Department of Health (NYCDOH) risk-assessment questionnaire for identifying children who should receive a TST. Questionnaire risk factors for TB infection were contact with a case of TB, foreign birth or travel to a TB-endemic area, contact with adults at high risk for TB (those who are infected with HIV, homeless, incarcerated, and illicit drug users), and HIV infection in a child. Contact with an adult with TB (OR 91.7, 95% CI 32.3–260.7), foreign birth or foreign travel (OR 14.8, 95% CI 6.7–32.7), and contact with a high-risk adult (OR 6.5, 95% CI 2.4–17.5) were independent risk factors for a positive TST result. Results for the full NYCDOH questionnaire were sensitivity 85.2%, specificity 86%, negative predictive value 99.8%, positive predictive value 5.4%, and OR 35.2 (95% CI 12.1–102.4). The data were interpreted as demonstrating that the NYCDOH questionnaire was a valid instrument for identifying children for TST. Children with ≥1 identifiable risk factor were 35 times more likely to have a positive TST result.

Screening Questionnaire for Risk Factors for LTBI

These studies have identified risk factors for LTBI in children. Based on these factors, a risk-assessment questionnaire was developed by the pediatric tuberculosis collaborative group to facilitate LTBI screening by pediatric healthcare providers (*19*). Pediatricians should ask the following questions when screening for risk factors of LTBI during the child's annual health maintenance visit (*19*). 1) Was your child born outside the United States? 2) Has your child traveled outside the United States? 3) Has your child been exposed to anyone with TB? 4) Does your child have close contact with a person who has had a positive TB skin test result? 5) Does your child spend time with anyone who has been in jail or a shelter, uses illegal drugs, or has HIV? 6) Has your child drunk raw milk or eaten unpasteurized cheese? 7) Does your child have a household member who was born outside the United States? 8) Does your child have a household member who has traveled outside the United States? A child or adolescent should be tested with TST only if ≥1 risk factor is present.

Challenges with Targeted Screening and LTBI Treatment Adherence

Despite the revised AAP recommendations for targeted TST and evidence for use of risk assessment, putting these guidelines into practice have presented some challenges. In 1996, the New York City Health Code was amended to require TST of only new entrants to secondary schools to reduce unnecessary screening of primary schoolchildren at low risk for LTBI. A study by Gounder et al. (*20*) assessed

adherence to this revised health policy change and showed that the proportion of new entrants into New York City's primary schools who were tested remained virtually unchanged after implementation of the health code amendment to discontinue testing of these children. In addition, older children who were more likely to be born in countries with high TB incidence and were at risk for LTBI were not tested.

Lack of clinician adherence to the LTBI screening guidelines has been shown in another study. Hsu et al. (*21*) found that most adolescents identified by risk-assessment questionnaire to be at risk for LTBI in 3 Boston schools were not adequately screened for TB infection. These studies show the necessity of programs to improve healthcare provider knowledge and acceptance of targeted TB screening guidelines. Research studies to assess the effect of such educational programs for clinicians on the targeted TB screening outcomes are needed. Future studies should also be conducted to compare the effectiveness of routine TST for all new high school entrants versus the use of the risk-assessment questionnaire in different populations.

Children diagnosed with LTBI must complete the prescribed regimen of isoniazid to maximize the protective effects of therapy. However, patient adherence to treatment for LTBI is low. Prevous studies have assessed different strategies to improve adherence to LTBI treatment (*22–25*). Morisky et al. (*22*) determined the effects of educational strategies to improve treatment of LTBI among adolescents in Los Angeles by randomly assigning them to a peer-counseling group, a group that received incentives, a combination of peer counseling and incentives, and a usual-care group. They found no difference in the rates of completion of LTBI treatment among the 4 groups.

Cass et al. (*23*) evaluated the effectiveness of a behavioral intervention, the Treasure Chest, to increase adherence to LTBI therapy in children. Each person received a monthly calendar with stickers and instructed to place a sticker on each day the medication was taken. When the completed calendar was returned, the child was allowed to select a toy from the Treasure Chest as a reward. Children who participated in the Treasure Chest program were 2.4 times more likely to complete therapy than those who did not. Several studies have also reported a significantly higher rate of completion of LTBI treatment among those high school students receiving directly observed therapy (*24*,*25*). Future studies should evaluate measures of adherence to LTBI therapy and other methods (educational efforts and various incentives) to improve adherence among different pediatric age groups and populations.

Logistic Aspects of Targeted Screening Programs

While the evidence for targeted TB testing exists and benefits of screening programs are clear, administrative logistics are of greater concern. The challenge for public health and school officials will be to develop a screening program that would avoid stigmatization of the at-risk group. One way to reduce stigmatization of the targeted group of children is to leave identification and screening of these children in the hands of their primary care providers. In New York City, every school year a medical information form that includes TST results is required for each currently or newly enrolled student (*20*). This form has recently been revised to reflect the targeted TB screening guidelines; however, not all schools have the updated forms.

To avoid stigmatization, targeted screening could also be accomplished by involving community organizations and local clinics that serve recent immigrants or the homeless population. For example, using community health workers who are members of the targeted communities may help eliminate language and cultural barriers in populations who are difficult to reach and screen for TB. Future studies are needed to assess the effectiveness of community health workers in improving the targeted screening of at-risk populations.

Conclusions

Several recent studies have shown the benefit of targeted TST and validated the use of risk-assessment questionnaires to identify children at increased risk for acquiring TB (*11–18*). These studies provide evidence in support of the targeted TST and recommendations favoring risk assessment over universal screening with a TST. Targeted TST and proper management of children with LTBI are essential components of the TB-elimination strategy promoted by the United States Public Health Service Advisory Council on the Elimination of Tuberculosis (*26*). Although targeted screening for LTBI in pediatric populations remains the current recommendation of CDC and AAP, clinician nonadherence to these guidelines results in overtesting children at low risk for LTBI and undertesting children at high risk for LTBI. In addition, the logistic issues with targeted screening programs are important. Public health measures must identify but not discriminate against high-risk populations. However, in practice, pediatric healthcare providers will continue to have a key role in identifying children at risk for LTBI. Thus, they should be familiar with risk factors for LTBI and screen children with TST only when ≥1 risk factor is present.

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Tuberculosis Transmission Attributable to Close Contacts and HIV Status, Malawi

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We conducted the first molecular study of tuberculosis (TB) to estimate the role of household contact and transmission from HIV-positive putative source contacts (PSCs) in a high HIV-prevalence area. TB patients in a long-term population-based study in Malawi were asked about past contact with TB. DNA fingerprinting was used to define clusters of cases with identical strains. Among 143 epidemiologically defined PSC-case pairs, fingerprinting confirmed transmission for 44% of household and family contacts and 18% of other contacts. Transmission was less likely to be confirmed if the PSC were HIV positive than if he or she were HIV negative (odds ratio 0.32, 95% confidence interval [CI] 0.14–0.74). Overall, epidemiologic links were found for 11% of 754 fingerprint-clustered cases. We estimate that 9%–13% of TB cases were attributable to recent transmission from identifiable close contacts and that nearly half of the TB cases arising from recent infection had acquired the infection from HIV-positive patients.

The HIV epidemic has dramatically increased tuberculosis (TB) incidence. The magnitude of this effect depends in part on the relative infectiousness of HIV-infected TB patients: they are less likely to have smear-positive disease and may be infectious for a shorter period than other patients since they have higher death rates and may seek health care earlier (*1*). Several studies have found that household contacts of HIV-positive patients had lower rates of *Mycobacterium tuberculosis* infection than those of HIV-negative patients, even after adjusting for sputum smear status of the cases and HIV status of the contacts (*2–4*), though other studies have found no differences in infection rates (*5–7*).

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DNA fingerprinting can be used to identify clusters of TB patients that share *M. tuberculosis* strains with identical patterns and to estimate when transmission occurred. To date, DNA fingerprinting studies comparing transmission from HIV-positive and HIV-negative patients have been small, and the differences have not been significant (*8*,*9*).

Studies can investigate sources of *M. tuberculosis* infection by seeking epidemiologic links within fingerprint-defined clusters or by comparing the DNA fingerprints of epidemiologically linked persons (*8–10*). In this study, we combine these 2 approaches to analyze the only long-term population-based molecular epidemiologic study of TB in an area with a high prevalence of HIV. Novel methods were used to estimate the proportion of TB in the population that is attributable to transmission from known contacts and from HIV-positive patients.

Methods

Since 1986, as part of the Karonga Prevention Study in northern Malawi, patients in whom TB was suspected have been identified by using enhanced passive surveillance. Project staff are based at peripheral clinics and the district hospital to examine anyone with chronic cough or enlarged lymph nodes. Patients in whom TB is suspected are also identified in the course of other studies, including household visits to TB patients, although in practice most patients come to the clinic or hospital. Sputum is taken for smear microscopic examination and culture, and material from lymph node biopsy specimens, ascites, and pleural fluid is also cultured when available (*11*). DNA fingerprinting has been carried out on cultures from all TB patients since late 1995 (*12*). Cultures that macroscopically resemble *M. tuberculosis* are sent to the Health Protection Agency Mycobacterium Reference Laboratory, London, United Kingdom, for species identification and drug sensitivity

testing. *M. tuberculosis* specimens are fingerprinted by using IS*6110* restriction fragment length polymorphism (RFLP), following standard procedures (*13*). Spoligotyping (*14*) is performed on strains with <5 bands on the RFLP pattern. Treatment follows Malawi National TB Control Programme guidelines. TB patients were tested for HIV after counseling and if consent was given. No antiretroviral treatment was available at the time of the study (*11*).

Since 1997, at the time of diagnosis, all TB patients have been asked about persons they knew who had had TB, either in their family or household (at any time) or among other acquaintances (in the last 5 years) (*15*). Details gathered on these putative source contacts (PSCs) allowed them to be identified within the project database: ≈90% of named PSCs who were said to have had TB in the district within the previous 5 years were confirmed as having been treated for TB. In addition, all persons seen in the district during long-standing epidemiologic studies are asked about their current and past residences and their parents, allowing genetic linkages and household histories to be constructed. PSCs identified only from the epidemiologic database were included in this study if they were first-degree relatives or half siblings of the patient or if they were documented as having lived in the same household as the patient at the time that the PSC had TB.

DNA fingerprints of cultures from all case-PSC pairs were compared by computer (Gelcompar 4.1, Applied Maths, Kortrijk, Belgium) and checked visually (*12*). Transmission was "confirmed" if the pair had identical strains, or if the RFLP patterns differed by 1 to 4 bands and the later strain was the first or only example of the new pattern in the dataset (*12,16*). Since >1 PSC was identified for some patients, the analysis was repeated excluding PSCs with smear-negative results or extrapulmonary disease, and, if >1 PSC was smear-positive, choosing the most likely source of the infection by selecting the most closely matched strains and the closest contacts (e.g., contacts within the household were considered closer than nonhousehold contacts). We have previously explored RFLP pattern evolution among the first 80 such pairs with smear-positive PSCs (to 2001) (*12*). Here, in a larger dataset, we explored risk factors associated with a named PSC being the confirmed source of transmission and estimate the proportion of TB in the population attributable to contact with a smear-positive household or other close family member and the relative contribution of HIV-positive and HIV-negative patients to transmission.

In addition, we estimated the proportion of RFLP-defined links that can be explained epidemiologically. Strains were defined as clustered if the RFLP pattern was shared by ≥2 patients. The proportion of patients in RFLP-defined clusters for whom epidemiologic links were known was calculated, and any variation with cluster size or band number was investigated. Assuming 1 index case per cluster, we calculated the proportion of secondary cases within clusters for which an epidemiologically linked source could be identified (*17*).

Statistical Analysis

Comparison of proportions used χ^2 tests, or exact tests when numbers were small. Odds ratios were calculated by using logistic regression. To calculate the proportion of cases in the population attributable to different types of contact (the population attributable fraction [PAF]), we adapted the formula PAF = p′(RR-1)/RR where p′ is the prevalence of the exposure (history of contact) in the cases, and RR is the relative risk of TB in those who are exposed compared to those who are not exposed (*18*). The expression (RR-1)/RR is algebraically equivalent to the risk difference percent, $(r_1–r_0)/r_1$, where r_1 is the risk in the exposed, and r_0 is the risk in the unexposed. We do not know RR, r_1, or r_0, but the risk difference percent is equivalent to the proportion of epidemiologically linked cases for which transmission from a PSC is confirmed (since this represents the proportion of cases in the exposed group that were caused by the exposure). PAF is thus calculated as the proportion of case-PSC pairs for which transmission was confirmed, multiplied by the prevalence of exposure (having a PSC) among the cases. To estimate the contribution of HIV-positive patients to onward transmission, we adjusted the relative probabilities of transmission being confirmed from HIV-positive and HIV-negative PSCs, by the proportion of smear-positive TB patients in the population who were HIV positive.

Ethics permission

Permission for the study was received from the Malawi National Health Sciences Research Committee and the ethics committee of the London School of Hygiene and Tropical Medicine.

Results

From late 1995 to early 2003, a total of 1,248 culture-positive TB patients were identified in Karonga District. Successful RFLP fingerprints were available on 1,194 isolates from 1,044 patients. After excluding 25 fingerprints because laboratory error was suspected (*12*), 1,029 patients had RFLP results: 74% were clustered (*19*). The isolates from 81 persons had <5 bands, and spoligotypes were available on 64 of these. HIV results were available for 61%, of whom 65% were positive.

Transmission Confirmation in Epidemiologically Defined Case-PSC Pairs

Fingerprints were available for 200 case-PSC pairs, of whom 51 had identical strains and 8 more had similar

Table 1. Comparison of *Mycobacterium tuberculosis* strains of index cases and putative source contacts (PSCs)

	Comparison of strains, no. (%)				
	Identical	1–4 bands different and first example of new strain	1–4 bands different and not first example	>4 bands different	Total
Total	51 (25.5)	8 (4.0)	15 (7.5)	126 (63.0)	200
Source of linking information					
Database only	9 (25.7)	1 (2.9)	5 (14.3)	20 (57.1)	35
History only	14 (16.5)	4 (4.7)	6 (7.1)	61 (71.8)	85
Both	28 (35.0)	3 (3.8)	4 (5.0)	45 (56.3)	80
Characteristic of PSC in case-PSC pairs					
Smear-positive pulmonary	48 (28.7)	7 (4.2)	15 (9.0)	97 (58.1)	167
Smear-negative pulmonary	2 (9.1)	1 (4.6)	0 (0.0)	19 (86.4)	22
Extrapulmonary	1 (9.1)	0 (0.0)	0 (0.0)	10 (90.9)	11
No. bands in RFLP* of PSC					
<5	6 (40.0)	1 (6.7)	0 (0.0)	8 (53.3)	15
5–10	17 (22.7)	6 (8.0)	8 (10.7)	44 (58.7)	75
>10	28 (25.5)	1 (0.9)	7 (6.4)	74 (67.3)	110

*RFLP, restriction fragment length polymorphism.

strains that were likely to be attributable to transmission between the 2 persons (Table 1). Transmission was no more likely to be confirmed if the information came from the patient's history only or from the epidemiologic database only, but was more likely if the information came from both sources ($p = 0.05$). Transmission was more likely if PSCs had smear-positive TB than if they had smear-negative or extrapulmonary TB ($p = 0.06$). Of the 7 pairs with confirmed matches and RFLP patterns with <5 bands, spoligotypes for both members of the pair were available for 3; they were identical for 2, and different for the third (a strain with 4 bands). The pair with different spoligotypes and a pair with similar but not identical RFLP patterns, with 1 band for the PSC and 4 bands for the patient (and missing spoligotypes), were excluded from further analyses.

When only smear-positive PSCs were used and the most likely source of transmission was selected, RFLP confirmation of transmission was much more likely for household and family PSCs (44%) than for other PSCs (friends, neighbors or colleagues, 18%, Table 2). Transmission was confirmed for 8 (62%) of 13 spouse pairs, and for 12 (48%) of 25 persons who nursed the sick patient or shared a sleeping dwelling with them. Transmission was less likely to be confirmed from male than from female PSCs, and less likely from HIV-positive PSCs than from HIV-negative PSCs (Table 2). The effect of sex of the PSC (odds ratio [OR] 0.39, 95% confidence interval [CI] 0.19–0.81) was reduced by adjusting for closeness of contact (OR 0.46, 95% CI 0.21–0.99) and was no longer significant after adjusting for HIV status of the PSC (OR 0.56, 95% CI 0.25–1.2). The effects of closeness and of HIV status of the PSC became slightly stronger when each factor was adjusted for: adjusted OR 4.6 (1.7–12.3) for family contacts and 4.1 (1.6–10.4) for household contacts, compared to other contacts; adjusted OR 0.32 (0.14–0.74) for HIV-positive contacts compared to HIV-negative contacts. These results were not altered by adjusting for degree of smear positivity of PSCs or for the other factors shown in Table 2. The results were similar if all index cases with <5 bands were excluded.

To estimate the origin of the infection in those for whom transmission from identified, PSCs was not confirmed, cases were classified as likely to be due to reactivation if the strain was the first or only example in the dataset and as recent infection if the strain was part of an existing cluster. For the patients without confirmed transmission from their PSCs, 33% had first/unique strains. In the whole dataset, the proportion of persons with first/unique strains was 39%, or 33% after excluding the first 2 years, in which first examples are more likely.

Proportion of TB Cases Due to Recognized Close Contact with a Smear-positive Patient

Of the 1,029 TB patients included in the study, 219 (21.3%) had at least 1 named family or household PSC with recorded smear-positive tuberculosis, and 86 other patients reported a PSC who was not identified in the database who may have had smear-positive disease. Overall, 177 (17.2%) of the patients had at least 1 PSC outside the family or household. Other patients either had no PSCs or none with smear-positive disease. Taking the proportion with transmission confirmed from family and household PSCs combined as 44.3% (Table 2) and the prevalence of exposure (at least 1 family or household PSC with smear-positive TB) as 21.3%, we estimate that $0.443 \times 0.213 = 9\%$ of TB case-patients in this population were attributable to recent transmission from identified smear-positive PSCs in their families or households. If the 86 additional PSCs are included, the estimate rises to 13%. Similarly, we estimate a PAF of 3.1% (0.182×0.172) for recent transmission from identified PSCs outside the family and household.

Table 2. Probability of transmission from a smear-positive putative source contact (PSC) being confirmed by restriction fragment length polymorphism, according to characteristics of case and PSC

Characteristic	Confirmed/ total pairs (%)	p	Odds ratio, 95% confidence interval (adjusted for closeness of contact)
Closeness of contact			
Same household	22/50 (44.0)		3.6 (1.4–9.3)
Close family, not household	17/38 (44.7)		3.5 (1.5–8.6)
Other	10/55 (18.2)	0.006	Referent
Time between diagnosis of disease in PSC and case-patient (mo)			
<12	21/62 (33.9)		Referent
12–23	13/36 (36.1)		1.1 (0.44–2.6)
≥24	15/45 (33.3)	1.0	0.88 (0.37–2.1)
Age of case-patient (y)			
<30	20/53 (37.7)		Referent
30–44	23/67 (34.3)		0.72 (0.32–1.6)
≥45	6/23 (26.1)	0.3 (trend)	0.48 (0.17–1.3)
Age of PSC (y)			
<30	20/49 (40.8)		Referent
30–44	21/63 (33.3)		0.93 (0.42–2.1)
≥45	8/31 (25.8)	0.2 (trend)	0.59 (0.19–1.8)
Sex of case-patient			
Female	28/81 (34.6)		Referent
Male	21/62 (33.9)	0.9	1.2 (0.56–2.4)
Sex of PSC			
Female	33/75 (44.0)		Referent
Male	16/68 (23.5)	0.01	0.46 (0.21–0.99)
HIV status of case-patient			
HIV negative	16/37 (43.2)		Referent
HIV positive	19/67 (28.4)	0.1	0.55 (0.23–1.3)
HIV status of PSC			
HIV negative	27/59 (45.8)		Referent
HIV positive	15/62 (24.2)	0.01	0.32 (0.14–0.74)
Drug resistance of PSC			
None	42/129 (32.6)		Referent
Isoniazid resistant	7/14 (50.0)	0.2	2.1 (0.66–6.8)
Smear positivity of PSC			
1+	5/18 (27.8)		Referent
2+	12/36 (33.3)		0.94 (0.26–3.5)
3+	12/43 (27.9)		0.89 (0.25–3.2)
4+	20/46 (43.5)	0.4	1.6 (0.46–5.4)

Proportion of TB Cases Attributable to Transmission from HIV-positive Patients

HIV-negative PSCs were twice as likely as HIV-positive PSCs to be confirmed by RFLP as sources of infection (46% vs. 24%), and this was seen both within the family and household (64% vs. 32%) and outside (23% vs. 12%). Overall, 61% of smear-positive TB patients were HIV positive. If we assume that the pattern of transmission from contacts is representative of the relative transmission from HIV-positive and HIV-negative patients in other settings, 45% of *M. tuberculosis* infections in this community are transmitted from HIV-positive patients: $(0.61 \times 0.24)/[(0.61 \times 0.24) + ([1–0.61] \times 0.46)]$.

Investigation of Clusters

Cluster sizes ranged from 2 to 37. The proportion of patients with clustered strains for whom epidemiologic links were identified is shown in Table 3. This proportion was no higher for strains with high band numbers than for those with <5 bands and did not vary consistently with cluster size. If we assume 1 index case per cluster, 623 of the 754 clustered cases were secondary. Of the 84 cases with epidemiologic links within RFLP-defined clusters, 52 were secondary, so sources of infection were identified for 8.3% (52/623) of secondary cases within clusters.

Conclusion

In Africa, case finding for TB is generally passive. Although being a household contact of a TB patient is a strong risk factor, in Africa as elsewhere (*15*,*20*), in high-incidence settings, most cases of TB are not attributable to household contact. This finding has been demonstrated in traditional epidemiologic studies (*21*) and more recently by using molecular techniques (*22*). The apparent importance

Table 3. Proportion of clustered strains with epidemiologic links

	No. with epidemiologic links within a cluster	Total with clustered strains	% with link
Total	84	754	11.1
No. bands			
≥5	72	679	10.6
<5	12	75	16.0
Cluster size (strains with ≥5 bands only)			
Cluster 2–4	18	187	9.6
Cluster 5–9	24	152	15.8
Cluster ≥10	30	340	8.8

of casual contact in TB transmission is not surprising since many people exposed to a small risk can account for more disease than a few exposed to a large risk (*23*).

DNA fingerprinting allows direct measurement of the proportion of cases with known exposure who acquired TB from that exposure. This proportion has varied from 95% in the Netherlands (*10*), 70% in San Francisco (*8*) and 71% elsewhere in the United States (*9*), to <50% in Cape Town, South Africa (*24*), and in our study. The studies varied in whether they included smear-negative PSCs, in the way contact was defined, and in whether similar but not identical RFLP patterns were included. Smear-negative PSCs were associated with a lower likelihood of confirmed transmission in our study and in the United States (*9*). Workplace contacts in the United States (*9*) and contacts outside the family and household in our study were much less likely to be confirmed as sources of infection. The inclusion of similar RFLP patterns that are the first example of their type will increase the proportion of confirmed transmissions, though with an increased risk of false attributions of the source of infection. Even identical strains may have other origins, particularly if the strain is common. On the other hand, actual transmission may not be recognized if different strains are seen in the PSC and case because of cross-contamination or other laboratory error or because the infection in the PSC was a mixture of strains.

This analysis, like all analyses to date of *M. tuberculosis* transmission based upon IS6*110* RFLP patterns, is based on the assumption that multiple infections are infrequent and thus that a single RFLP-defined strain reflects the infection status of a patient. A recent study from South Africa has hinted that multiple infections may be more frequent than previously assumed (*25*). If this is the case, then we (and all previous RFLP-based studies) have underestimated the proportions of transmission occurring within households. This assumption should not affect our estimates of relative transmission from HIV-positive and HIV-negative patients.

A lower proportion of confirmed transmission from identifiable PSCs is expected in high-incidence settings. As the incidence of TB decreases in a population, patients are increasingly concentrated in high-risk groups with particular risk factors for disease. Close contacts of patients may share many risk factors. As the risk of a close contact of a TB patient having had TB increases relative to the risk of a casual contact having had TB, the proportion of TB due to transmission from a close contact will also increase. In our study, 44% of patients with a smear-positive PSC in their household within the previous few years appear to have acquired their infection from that person. The source of the infections in persons without confirmed transmission is unknown, but two thirds of patients were part of existing clusters so the infections probably were recently acquired locally.

In Cape Town, the proportion of identifiable household contacts with confirmed transmission was similarly low (*24*). In that study, the proportion of TB in the community attributable to transmission within the household (from smear-positive or smear-negative contacts) was estimated at 19%. In our study, by using different methods, we estimated that 9%–13% of TB cases were attributable to transmission from smear-positive PSCs within the household or close family, and 3% from other named sources. The low proportion of cases with identified sources of infection is corroborated by conventional epidemiologic studies in this and other populations (*15*,*21*).

The low proportion of TB attributable to identifiable links is also supported by the similarly low proportion of persons in clusters who can be linked epidemiologically, both in our study and studies in South Africa (*22*) and India (*26*). In our study, the epidemiologic links were established independently of the molecular data; further links might have been found by detailed investigations of particular clusters (*10*), but many of these more-difficult-to-define links represent casual contact. Identifiable links have been found for a higher proportion of clustered patients in low-incidence settings (*10*,*27*,*28*), but, excluding the casual links, the proportion is still <50%.

One reason for the particularly low proportion of confirmed transmission in our study is the high prevalence of HIV and the effect of HIV on transmission. Our study is the first to demonstrate lower infectiousness of HIV-infected TB patients by DNA fingerprinting of epidemiologically linked case-contact pairs. The lower rate of transmission

persisted after adjusting for degree of smear positivity. Although HIV status was not available for all patients, this factor should not bias this estimate.

Extrapolating the results from case-PSC pairs to the community assumes similar relative transmission patterns, but is reasonable since HIV-positive patients had similarly reduced transmission within families and households and outside them. The lower infectiousness of HIV-positive patients does not mean that they have a minor role in TB transmission, since nearly two thirds of TB patients are HIV positive. It does, however, help limit the extent of the HIV-related increase in TB in the population (*1*,*11*,*29*).

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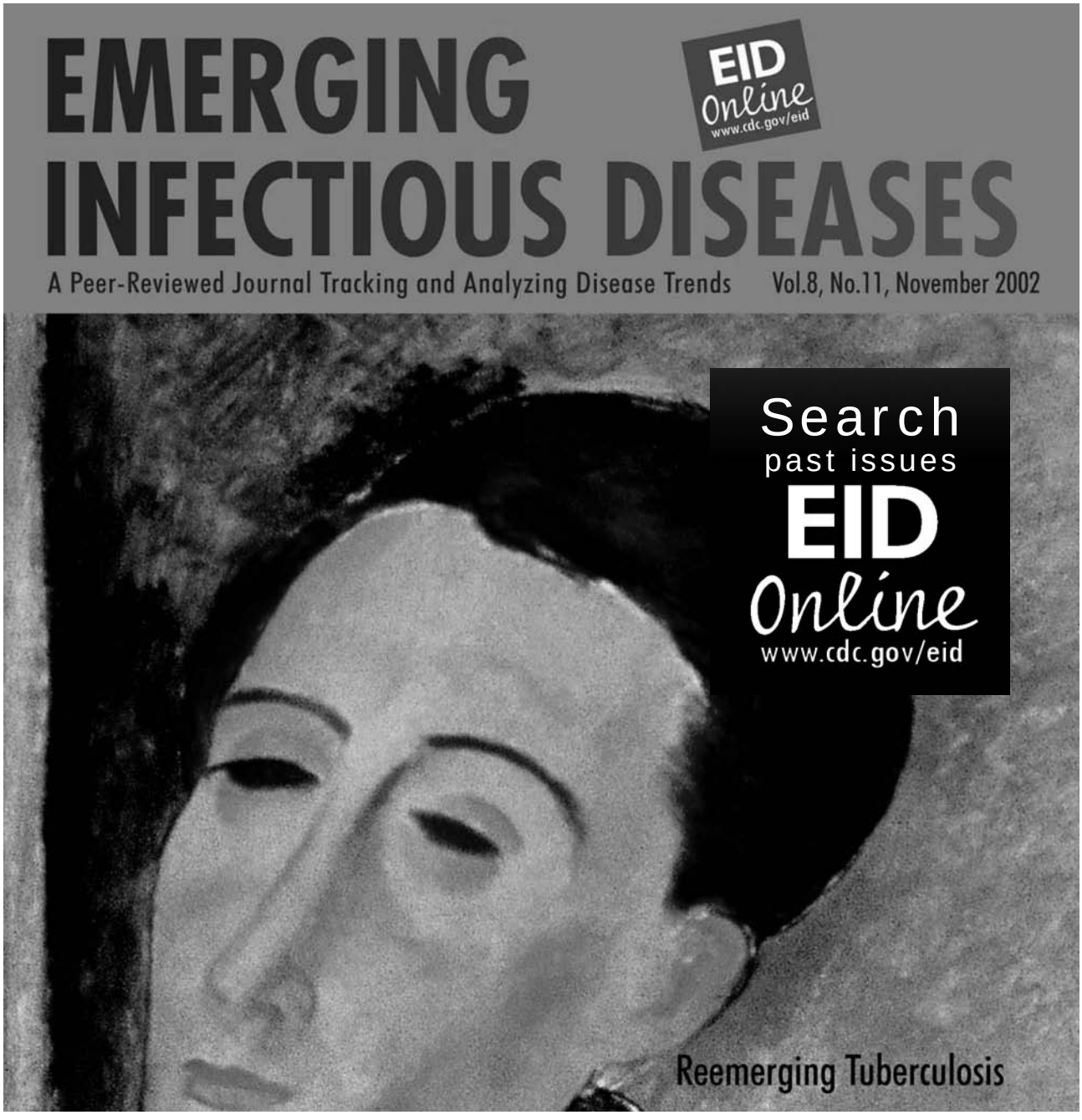

Beijing/W Genotype *Mycobacterium tuberculosis* and Drug Resistance

European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis*[1]

Beijing/W genotype *Mycobacterium tuberculosis* is widespread, may be increasing, and may have a predilection for drug resistance. Individual-level data on >29,000 patients from 49 studies in 35 countries were combined to assess the Beijing genotype's prevalence worldwide, trends over time and with age, and associations with drug resistance. We found 4 patterns for Beijing/W genotype tuberculosis (TB): 1) endemic, not associated with drug resistance (high level in most of East Asia, lower level in parts of the United States); 2) epidemic, associated with drug resistance (high level in Cuba, the former Soviet Union, Vietnam, and South Africa, lower level in parts of Western Europe); 3) epidemic but drug sensitive (Malawi, Argentina); and 4) very low level or absent (parts of Europe, Africa). This study confirms that Beijing/W genotype TB is an emerging pathogen in several areas and a predominant endemic strain in others; it is frequently associated with drug resistance.

The *Mycobacterium tuberculosis* genotype family known as "Beijing/W," "W-Beijing," or "Beijing" is widespread (*1*–*3*). Described in 1995 as the prevalent genotype in East Asia (*4*), >80% of strains from the Beijing area were of this type. The multidrug-resistant W strain is a member of the family. We use "Beijing" for the whole genotype family.

Researchers are concerned that the Beijing genotype may have a predilection for developing drug resistance (*5*) and may be spreading worldwide, perhaps as a result of increased virulence (*6*). A systematic review of the published literature in 2002 concluded that although Beijing genotype tuberculosis (TB) was widespread, associations with drug resistance varied, and little information on time trends was available (*2*).

The review highlighted the problems of relying on published literature: varying strain definitions; reporting bias; and limited information on selection criteria, population subgroups, age groups, or time trends. As part of the European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis, we have combined available datasets, using a common strain definition and individual-level data.

Methods

Studies for inclusion were identified from the systematic review and from contacting members of the European Concerted Action and authors of relevant articles published since the review. We aimed to include as many studies as possible in which the proportion of TB caused by the Beijing genotype could be ascertained in an unbiased way. Studies could represent all or random samples of patients in an area, hospital, or laboratory. Studies limited to outbreaks, drug-resistant isolates, or of <30 patients were excluded. A study description and individual patient data that included at least the year the case was diagnosed and the genotype were required.

Strain Classification

Three methods identify Beijing genotype strains: spoligotyping (*7*), IS*6110* restriction fragment length polymorphism (RFLP) (*8*), and region A RFLP (*9*). The typical Beijing spoligotype shows hybridization to spacers 35–43. Beijing-like patterns with <9 spacers (but not solely spacers 37–38, which represents *M. microti*), were included (*10*).

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By using IS*6110* RFLP, fingerprints are compared to 19 patterns representative of the Beijing genotype (https://hypocrates.rivm.nl/bnwww/index.html). With standard techniques, allowing 1% position tolerance and classifying all matches >80% as Beijing, these patterns have 96%–100% sensitivity and 98%–100% specificity to detect Beijing strains, taking spoligotyping as the accepted standard (*10*). Sensitivity is increased by spoligotyping strains with RFLP patterns that match 75%–80% to the reference strains. The third technique uses a characteristic IS*6110* insertion in region A. This method has 100% sensitivity and 98% specificity compared with spoligotyping (*10*).

Analysis

The proportion of Beijing genotype strains in each study was calculated overall and after excluding immigrants. The proportion of Beijing genotype in immigrants was examined by place of birth. Time trends were examined directly and by examining trends with age; an association with younger age groups would suggest that the proportion of TB attributable to the Beijing genotype was increasing. Associations with drug resistance were examined, after immigrants were excluded, with and without excluding patients with previous TB. For pooled analyses, heterogeneity in the associations between studies was examined, and the results presented are adjusted for study.

Results

Data were received from 49 studies representing 29,259 TB patients in 35 countries, including 11 studies from the systematic review (*2*); other studies in the review had no individual patient data available, used nonstandard case definitions, or researchers declined to participate. Other studies were contributed by members or contacts of the Concerted Action or were identified from subsequently published studies. Details of all included studies are shown in online Appendix Table 1 (available from http://www.cdc.gov/ncidod/EID/vol12no05/05-0400_app.htm).

The proportion of tuberculosis due to the Beijing genotype in the included studies is shown in online Appendix Table 2 (available from http://www.cdc.gov/ncidod/EID/vol12no05/05-0400_app.htm#table2).

Overall, 9.9% had the Beijing genotype. In Western Europe and the Czech Republic, the proportion was low: <6% of cases among nonimmigrants. In sub-Saharan Africa, the proportion was low except in Cape Town, South Africa. In Latin America, data were only available from Argentina and Brazil; both studies found <1% of TB cases were caused by Beijing genotype. In North America and the Caribbean, the proportion was higher (8%–14%). In the former Soviet Union the proportion was high: 45%–56% in Russia and 29% in Estonia. The proportion was low in India (1%), higher in Bangladesh (7%), and increased further east: >50% in many parts of Southeast and East Asia.

Analyses by region of birth showed similar patterns (Table 1). Beijing genotype strains were rare (0.5%) among immigrants from Eastern Europe other than the former Soviet Union; most came from the former Yugoslavia. The Beijing genotype was much less common among immigrants from the Indian subcontinent (3.4%) than among those from Southeast Asia (19%) or East Asia (58%). Beijing genotype strains were uncommon among immigrants from North Africa (3.0%), the Middle East (5.2%), and sub-Saharan Africa (2.2%, including 50 [2.1%] of 2,427 persons from Somalia). Among Middle Eastern immigrants, Beijing genotype was found in 6 (1%) of 620 persons from Turkey but in 8 (9.9%) of 81 from Afghanistan.

Time Trends

Time trends were analyzed among nonimmigrants within individual studies with ≥3 years of data (Table 2). (Studies from France, Iran, Thailand, Vietnam, and Spain are excluded because of small numbers in some years or absence of Beijing genotype strains.)

All Western European sites except London showed a slight increase in Beijing strains over time, but this finding was only significant in the Netherlands. Combining data for Western Europe, the odds ratio (OR), adjusted for study, for having the Beijing genotype in the later period compared to the earlier period was 1.5 (95% confidence interval [CI] 1.2–1.9). This figure was unchanged after adjusting for age. The trend was similar after excluding the Netherlands (adjusted OR 1.7, 95% CI 0.96–3.1).

In St. Petersburg, Okayama, Buenos Aires, São Paulo, and San Francisco, no significant change occurred over time, but the studies only covered a few years. In Cape Town and Malawi, significant increases occurred over time and were unchanged after adjusting for age.

Trends with Age

Trends with age for studies with ≥3 cases of Beijing genotype TB among nonimmigrants are summarized in Table 3. Most Western European studies found the highest proportion of Beijing genotype TB in the youngest age groups. Overall, for Western Europe, compared to those age ≥50 years, the OR, adjusted for study, of having the Beijing genotype was 1.2 (0.87–1.6) for those 30–49 years of age, and 2.4 (95% CI 1.8–3.3) for those <30 years of age, $p_{trend}<0.001$. Excluding the Netherlands, the trend was stronger: adjusted OR 2.2 (95% CI 1.1–4.2) for those 30–49 years of age and 3.9 (95% CI 1.9–7.9) for those <30 years of age, $p_{trend}<0.001$.

In Russia and Estonia, Beijing genotype strains were more common in younger patients, and the trend was

Table 1. Proportion of tuberculosis patients due to the Beijing genotype by region of birth

Region	All patients, Beijing/total (%)	Immigrants only, Beijing/total (%)
Western Europe	272/9,496 (2.9)	10/353 (2.8)
Central and Eastern Europe	4/780 (0.5)	3/562 (0.5)
Former Soviet Union	244/590 (41.4)	25/106 (23.6)
Middle East	62/1,165 (5.3)	56/1,084 (5.2)
North Africa	30/991 (3.0)	30/991 (3.0)
Sub-Saharan Africa	275/6,816 (4.0)	86/3,881 (2.2)
Indian subcontinent	46/1,291 (3.6)	38/1,111 (3.4)
Southeast Asia	711/2,192 (32.5)	154/811 (19.0)
East Asia	1,032/1,712 (60.3)	213/370 (57.6)
Latin America	29/1,421 (2.0)	21/457 (4.6)
Caribbean	31/320 (9.7)	5/109 (4.6)
North America	28/275 (10.2)	1/15 (6.7)
Australasia	1/4 (25.0)	1/4 (25.0)

significant in St. Petersburg (p = 0.02). Overall, compared to those ≥50 years of age, the study-adjusted OR was 1.1 (95% CI 0.70–1.8) for those 30–49 years of age and 1.7 (95% CI 1.1–2.9) for those <30 years of age, $p_{trend} = 0.02$.

The African studies that found any Beijing strains noted a higher proportion in younger persons than in older persons. This difference was not significant in individual studies but was when studies were combined: study-adjusted OR, 1.9 (95% CI 1.1–3.4) for those 30–49 years of age and 2.1 (95% CI 1.2–3.7) for those <30 years of age, compared to those ≥50 years of age, $p_{trend} = 0.03$.

Among nonimmigrants in US studies, no significant trend occurred with age, either individually or overall. In Cuba, Beijing genotypes were more common in younger persons than in older persons in the larger study and overall ($p_{trend} = 0.06$). In Buenos Aires and São Paulo, all Beijing genotype–infected patients were <30 years of age (p = 0.002).

Most Asian studies showed no association with age, but trends were seen in Bangladesh, Vietnam, and Hong Kong. In Vietnam, Beijing genotype was more common in younger patients in all 4 studies: overall, compared to those ≥50 years of age, the study-adjusted OR was 1.5 (95% CI 1.0–2.2) for those 30–49 years of age and 2.7 (95% CI 1.7–4.2) for those <30 years of age, $p_{trend}<0.001$. In Hong Kong, Beijing genotypes were least common in patients <30 years of age.

Drug Resistance

Studies with drug resistance data for all or most patients and with ≥3 Beijing genotype TB patients among nonimmigrants are summarized in online Appendix Table 3 (available at http://www.cdc.gov/ncidod/EID/vol12no05/05-0400_app.htm#table3). In the Western European studies, with the exception of inner London, resistance was more common among Beijing genotype strains than among other strains. Beijing genotype was significantly associated with resistance in Denmark (rifampin and ethambutol), Finland (rifampin and streptomycin), and the Netherlands (streptomycin). Overall, the study-adjusted OR for the association of Beijing genotype and resistance among nonimmigrants in Western Europe was 1.8 (95% CI 1.2–2.7) for any drug, 1.7 (95% CI 0.95–2.9) for isoniazid, 4.0 (95% CI 1.4–11.9) for rifampin, 2.3 (95% CI 1.4–3.7) for streptomycin, 3.0 (95% CI 0.38–23.2) for ethambutol, and 4.2 (95% CI 1.2–14.7) for multidrug resistance (i.e., resistance to at least isoniazid and rifampin). Of the Western European studies, only those from Denmark, Hamburg, the Netherlands, and London had data on previous treatment. After patients who had previously received treatment were excluded, the associations in the Netherlands and Denmark persisted, and the adjusted combined ORs were similar to those overall but with wider CIs (e.g., 1.6, 95% CI 1.0–2.6 for any drug resistance).

Table 2. Trends in proportion of tuberculosis cases due to the Beijing genotype over time among nonimmigrant populations*

Study	Period	Earlier period,† Beijing/total (%)	Later period,† Beijing/total (%)	OR (95% CI) for change/y	p for linear trend by y
Western Austria	1993–2004	2/363 (0.6)	5/310 (1.6)	1.2 (0.9–1.5)	0.2
Denmark	1992–2001	7/885 (0.8)	10/774 (1.3)	1.1 (0.9–1.3)	0.4
Finland	2000–2002	2/414 (0.5)	11/705 (1.6)	1.7 (0.9–3.5)	0.1
The Netherlands	1993–2002	91/1,862 (4.9)	111/1,607 (6.9)	1.1 (1.0–1.1)	0.004
Western Sweden	1999–2002	0/34 (0.0)	3/43 (7.0)	3.1 (0.6–15)	0.2
London, UK	1995–1997	9/200 (4.5)	1/73 (1.4)	0.7 (0.3–1.8)	0.4
St. Petersburg, Russia	1999–2001	66/120 (55.0)	67/116 (57.8)	1.0 (0.7–1.3)	0.9
Cape Town, South Africa	1992–1998	60/473 (12.7)	80/374 (21.4)	1.2 (1.1–1.3)	<0.001
Karonga, Malawi‡	1996–2003	12/460 (2.6)	32/570 (5.6)	1.2 (1.0–1.4)	0.03
San Francisco, USA	1998–2000	6/50 (12.0)	6/59 (10.2)	1.0 (0.5–2.1)	1.0
Buenos Aires, Argentina	1998–2001	1/188 (0.53)	4/424 (0.94)	1.0 (0.4–2.3)	1.0
São Paulo, Brazil	2000–2002	2/268 (0.75)	1/114 (0.88)	1.0 (0.2–4.3)	1.0
Okayama, Japan	2000–2002	42/56 (75.0)	61/86 (70.9)	0.8 (0.5–1.3)	0.4

*OR, odds ratio; CI, confidence interval.
†For each study, the period was split into 2 parts, earlier and later.
‡Includes immigrants from neighboring countries.

Table 3. Proportion of tuberculosis cases caused by the Beijing genotype by age group of patients*

Study	Age <30 y, Beijing/total (%)	Age 30–49 y, Beijing/total (%)	Age ≥50 y, Beijing/total (%)	p for trend
Western Europe				
Western Austria	2/89 (2.3)	45/214 (1.9)	1/370 (0.3)	0.05
Denmark	4/210 (1.9)	6/623 (1.0)	7/826 (0.9)	0.3
Finland	2/35 (5.7)	5/128 (3.9)	6/931 (0.6)	0.002
The Netherlands	70/703 (10.0)	47/993 (4.7)	85/1773 (4.8)	<0.001
Western Sweden	1/5 (20.0)	1/7 (14.3)	1/65 (1.5)	0.05
United Kingdom				
Inner London	1/41 (2.4)	2/67 (3.0)	2/55 (3.6)	0.7
London	6/86 (7.0)	1/104 (1.0)	3/83 (3.7)	0.2
Eastern Europe				
Estonia	14/43 (32.6)	25/96 (26.0)	15/52 (28.9)	0.7
Russia				
St. Petersburg	74/112 (66.1)	61/111 (55.0)	19/45 (42.2)	0.02
Archangel†	13/25 (52.0)	32/77 (41.6)	8/16 (50.0)	0.8
Middle East				
Iran	2/20 (10.0)	2/25 (8.0)	1/26 (3.9)	0.4
Sub-Saharan Africa				
Malawi‡	19/341 (5.6)	21/522 (4.0)	4/167 (2.4)	0.08
South Africa: Cape Town	51/299 (17.1)	77/434 (17.7)	11/111 (9.9)	0.2
Zimbabwe: Harare	3/94 (3.2)	1/102 (1.0)	0/16 (0.0)	0.2
North America				
United States				
New Jersey	1/18 (5.6)	11/62 (17.7)	3/71 (4.2)	0.2
San Francisco	1/21 (4.8)	7/58 (12.1)	4/30 (13.3)	0.4
Caribbean				
Cuba				
Not Havana	10/48 (20.8)	6/42 (14.3)	6/70 (8.6)	0.06
Havana	1/11 (9.1)	2/21 (9.5)	1/19 (5.3)	0.7
Latin America				
Argentina: Buenos Aires	5/255 (2.0)	0/224 (0.0)	0/103 (0.0)	0.05
Brazil: São Paulo	3/144 (2.1)	0/187 (0.0)	0/51 (0.0)	0.1
Indian subcontinent				
Bangladesh†	3/20 (15.0)	4/42 (9.5)	0/35 (0.0)	0.03
Southeast Asia				
Indonesia: Jakarta	13/45 (28.9)	14/33 (42.4)	5/12 (41.7)	0.2
Malaysia	17/93 (18.3)	20/129 (15.5)	25/162 (15.4)	0.6
Thailand: Bangkok	33/64 (51.6)	41/88 (46.6)	24/52 (46.2)	0.5
Vietnam				
Hanoi†	11/15 (73.3)	17/26 (65.4)	9/23 (39.1)	0.03
Ho Chi Minh City†	94/147 (64.0)	134/265 (50.6)	35/87 (40.2)	<0.001
Ho Chi Minh City	13/21 (61.9)	17/40 (42.5)	4/14 (28.6)	0.04
Tien Giang	4/7 (57.1)	11/27 (40.7)	13/26 (50.0)	1.0
East Asia				
China				
Shanghai and other areas†	5/5 (100.0)	10/14 (71.4)	16/24 (66.7)	0.2
Henan	10/19 (52.6)	7/9 (77.8)	16/21 (76.2)	0.2
Hong Kong†	95/151 (62.9)	149/197 (75.6)	112/152 (73.7)	0.04
Japan: Okayama	9/12 (75.0)	19/25 (76.0)	75/105 (71.4)	0.7
Mongolia	50/95 (52.6)	42/63 (66.7)	5/10 (50.0)	0.3
Taiwan†	25/47 (53.2)	36/83 (43.4)	126/291 (43.3)	0.3

*Studies with ≥3 cases of Beijing genotype tuberculosis in nonimmigrants included.
†Immigration status not known.
‡Immigrants from neighboring countries included.

In Russia and Estonia, Beijing genotype was strongly associated with resistance to all tested drugs. None of the patients in Estonia had been previously treated. In the Archangel Oblast, the association persisted after previously treated patients were excluded, but in St. Petersburg only the association with isoniazid resistance remained significant. In Cuba, Beijing genotype was associated with streptomycin resistance in both studies, and this association persisted after previously treated patients were excluded.

In Malawi and Zimbabwe, none of the Beijing genotype isolates was drug resistant. In Cape Town, 14 (35%) of the 40 Beijing isolates that were tested were drug resistant, but the resistance of most Beijing isolates and of the other isolates was unknown.

In the Asian studies, only those in Bangladesh, Vietnam, and Taiwan found more drug resistance in Beijing genotype strains. In Bangladesh, 99% of the patients had previously received treatment for TB. In Vietnam, the results were little changed by excluding the few previously treated patients. In Taiwan, previous treatment was unknown. Two studies found that Beijing genotypes were less commonly drug resistant. In China, Beijing genotypes were less likely to exhibit ethambutol resistance; no information was available on previous treatment. In Malaysia, among patients without previous treatment, 1 (2%) of 48 isolates from patients with the Beijing genotype and 33 (13%) of 252 isolates from patients with other genotypes were resistant to any drugs ($p = 0.03$).

Other Associations

In most studies, the proportion of nonimmigrants with the Beijing genotype was similar for men and women. In Japan, the proportion was higher among men, and in Malawi, it was higher among women. Only 23 studies had data on HIV status in nonimmigrants, and of these, 13 found no Beijing genotype, no HIV-positive patients, or information was lacking on HIV status of the patients with Beijing genotype. In the 10 remaining studies (inner London, Lyon, the Netherlands, Tuscany, San Francisco, Cuba [both studies], Buenos Aires, Malawi, and Ho Chi Minh City), no association was found between HIV status and Beijing genotype.

No significant association was found between strain type and site of tuberculosis (pulmonary or extrapulmonary) in any of the 20 studies in which this information was available and both types of tuberculosis were included. In Cuba, outside Havana, and in the Archangel Oblast patients with recurrent TB were more likely than patients in their first episode of disease to have the Beijing genotype, but these associations were lost after adjusting for drug resistance. No associations with previous TB were found in any of the other 17 studies for which information was available, but the numbers of recurrent cases were often small.

Discussion

In this study, we have brought together published and unpublished data to document the spread of Beijing genotype tuberculosis worldwide. Little information was available from many countries including most of the Americas, Eastern Europe, North Africa, the Middle East, and Australasia. All eligible studies were requested, whether Beijing genotypes were found or not, and within the included studies, the proportion with Beijing genotype should be representative of those settings. The individual-level data allowed comparable analyses in all sites and pooled analysis within regions. This study complements the spoligotype database (*3*), which includes only studies that used spoligotyping and is more inclusive and less detailed epidemiologically. The database shows a similar global distribution of the Beijing genotype to that described here.

The proportion of TB attributable to the Beijing genotype is variable: high in Asia, apart from the Indian subcontinent, increasing further east; low in parts of Africa, Latin America, and Western Europe; intermediate in the United States and Cuba; low in Eastern Europe (other than the former Soviet Union); low in the Middle East (including <1% in a recent study from Tehran [*11*]). In Western Europe, Beijing genotype is more common among immigrant TB patients than among indigenous patients. The proportion of Beijing genotype TB among nonimmigrants may reflect the importance of immigrants to the total TB prevalence in these countries as well as the origin of these immigrants. Immigrants accounted for >50% of TB cases in London, the Netherlands, France, Denmark, Sweden, and Hamburg, compared to 25% of cases in Italy, 24% in Austria, 8% in Finland, and 4% in Spain.

Using information from time and age group trends, we found that an increasing proportion of TB is due to Beijing genotype strains in Western Europe, southern Africa, and the former Soviet Union. We found little evidence of increase in Asia, except in Vietnam and Bangladesh.

Strong associations with drug resistance have been found in the former Soviet Union, Cuba, and Vietnam. The combined data for Western Europe suggest an association there. No association was found in a large study in Malawi or in most of the Asian studies.

When the data on trends and drug resistance presented here and from other studies are combined, the results suggest that the distribution of Beijing genotype TB has several patterns (Figure). The Beijing genotype probably originated in the Beijing region of China (*1*,*4*); it was found in 90% of stored biopsy specimens in the 1950s, and this proportion has not changed over time (*12*). Beijing strains appear to have spread and become established as the predominant *M. tuberculosis* genotype in much of East and Southeast Asia, so little evidence of increase was found. In

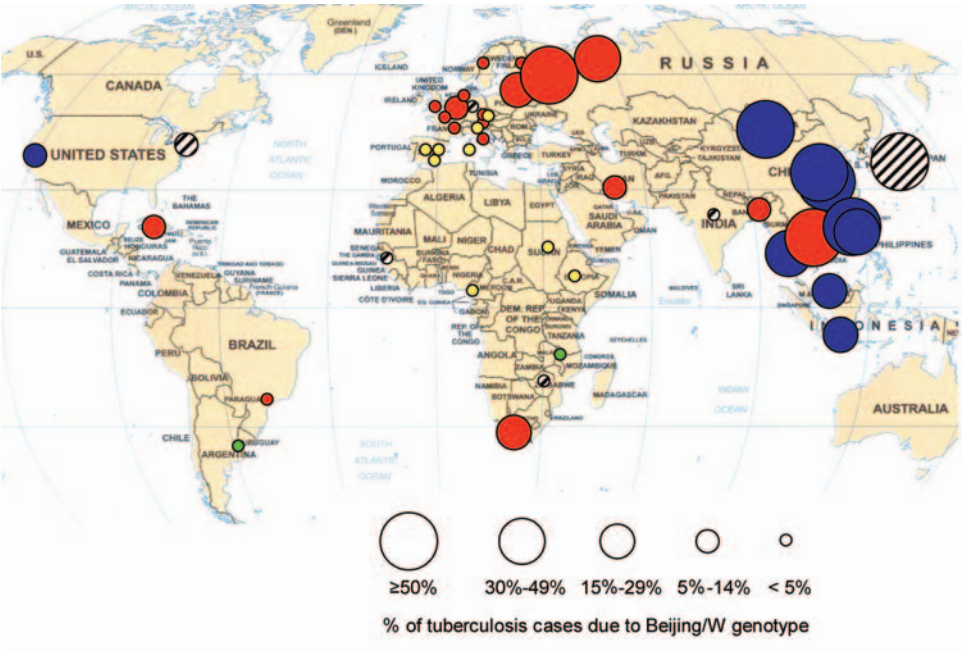

Figure. Distribution of Beijing genotype tuberculosis (TB). Size of circle indicates percentage of TB cases due to Beijing genotype; color in circle indicates drug sensitivity and distribution trend. Blue, pattern 1 (stable, no association with drug resistance); red, pattern 2 (increasing, associated with drug resistance); green, pattern 3 (increasing, drug sensitive); yellow, pattern 4 (absent); striped, trend or association with drug resistance not known.

these areas, the Beijing genotype appears to be endemic and not associated with drug resistance (pattern 1).

In certain areas, including the former Soviet Union (*13*), Cuba, and Cape Town, epidemic spread was found, which was associated with drug resistance (pattern 2). Vietnam and Bangladesh follow this pattern, unlike most other Asian countries. Recent Indian studies suggest that India may also fit pattern 2 (*14*,*15*). In Taiwan, the association with drug resistance was not confirmed in a larger sample in 2003 (unpub. data), which suggests that it follows pattern 1. In parts of Western Europe, although the Beijing genotype remains uncommon, it appears to be increasing and is associated with drug resistance (pattern 2).

In the United States, the pattern is mixed. Nonimmigrant patients in San Francisco fit pattern 1: no association with drug resistance and no evidence of time trends. In this area, most Beijing isolates came from Asian immigrants, among whom no association was found between Beijing genotype and drug resistance. In the New Jersey study, no data on drug resistance were available, but a previous study in this area found that most Beijing isolates from nonimmigrants were pansusceptible (*1*,*16*). The age distribution does not suggest recent increase, which fits pattern 1. In contrast, the spread of the multidrug-resistant W strain in New York and beyond during the 1990s has been well documented (*17*–*19*). Other published studies from the United States confirm that the Beijing genotype is widespread but do not report drug resistance or trends (*20*–*23*).

In Malawi, an increase in the Beijing genotype over time was documented, but with drug sensitive strains (pattern 3). Argentina may fit this pattern, and spread of drug-sensitive Beijing genotype TB has been described in Gran Canaria (*24*). The final pattern (pattern 4) is of very low level or absent Beijing genotypes, as seen in parts of Africa and Europe.

The wide distribution of the Beijing genotype could be attributable to a founder effect or random drift, though these mechanisms would be unlikely to account for recent increases in multiple settings. The distribution could reflect particular stability of the genetic markers used to identify the genotype. High levels and epidemic spread may suggest that it transmits more easily or is more virulent than other strains. In vitro and animal studies have suggested increased multiplication or virulence for some Beijing strains (*6*,*25*) but not others (*26*). In Vietnam, the Beijing genotype was associated with treatment failure and relapse (*27*), but we found no such association. In Indonesia, patients with the Beijing genotype had a similar clinical picture to other TB patients for almost all parameters studied (*28*). In the Netherlands, the appearance on chest radiograph was similar for patients infected with Beijing genotype and for other TB patients (*29*). In Malawi, the Beijing genotype was not associated with death or transmissibility (*30*).

External factors may select for Beijing strains. In the former Soviet Union and the United States, spread has been associated with prisons and with high rates of drug resistance (*13*,*17*,*31*,*32*). In Mongolia, data were also available from prisoners. They had a higher proportion of Beijing genotype than did other patients, 46 (82%) of 56 compared to 97 (58%) of 168, p = 0.001, and a higher

prevalence of drug resistance. Population movements (*33*), for example, from the former Soviet Union into Western Europe and through Afghanistan, may account for spread, recent increases, and the association with drug resistance (*34*).

Beijing genotypes may have a particular propensity to acquire drug resistance. Mutations in putative mutator genes have been found in Beijing genotypes, which suggests adaptability (*5*), but no increase in the rate of acquisition of resistance to rifampicin was found in in vitro studies (*35*). Once established, resistance could encourage spread if it delays effective treatment. Although the fitness of resistant strains is slightly reduced, this may be less marked for Beijing strains (*36*).

Conclusion

This study has confirmed that Beijing genotype *M. tuberculosis* is an emerging infection in many parts of the world and is a highly endemic pathogen in other areas. Its association with drug resistance, sometimes at high levels, in a number of settings, underlines its importance. The reasons for its apparent success are not well understood but may depend on human population movements as well as on any intrinsic factors.

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Isoniazid Preventive Therapy and Risk for Resistant Tuberculosis

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In the context of tuberculosis (TB) resurgence, isoniazid preventive therapy (IPT) is increasingly promoted, but concerns about the risk for development of isoniazid-resistant tuberculosis may hinder its widespread implementation. We conducted a systematic review of data published since 1951 to assess the effect of primary IPT on the risk for isoniazid-resistant TB. Different definitions of isoniazid resistance were used, which affected summary effect estimates; we report the most consistent results. When all 13 studies (N = 18,095 persons in isoniazid groups and N = 17,985 persons in control groups) were combined, the summary relative risk for resistance was 1.45 (95% confidence interval 0.85–2.47). Results were similar when studies of HIV-uninfected and HIV-infected persons were considered separately. Analyses were limited by small numbers and incomplete testing of isolates, but findings do not exclude an increased risk for isoniazid-resistant TB after IPT. The diagnosis of active TB should be excluded before IPT. Continued surveillance for isoniazid resistance is essential.

Tuberculosis (TB) has reemerged as a major threat to global public health. Its incidence is rising, particularly in countries with a high HIV prevalence (*1*). HIV-infected persons have an increased risk for reactivated latent TB infection (*2*), of having new TB infection progress rapidly to active disease (*3*,*4*), and of dying during a TB episode (*5*).

Since current TB control methods appear inadequate to prevent the rise in TB incidence among HIV-infected persons in settings with high TB prevalence (*6*), additional measures are required. Studies in the late 1980s and 1990s found that TB "preventive therapy" (treatment of latent TB infection) reduced TB incidence among HIV-infected persons, at least among those with positive tuberculin skin test results (*7*). However, despite recommendations from the World Health Organization (WHO) and the Joint United Nations Programme on HIV/AIDS in 1998 (*8*), TB preventive therapy has not been widely adopted. One obstacle to more widespread use is the concern that using isoniazid monotherapy to treat latent TB infection could promote isoniazid-resistant TB; a literature review in 1970 concluded that, since the introduction of isoniazid in 1952, no evidence existed to support this conclusion (*9*).

Since then, a number of placebo-controlled trials of isoniazid preventive therapy (IPT) have been conducted, mostly among HIV-infected persons. We carried out a systematic review of studies (in both the pre-HIV and the HIV era) that compared those who received IPT to an untreated group and reported data on resistance to isoniazid, aiming to assess the effect of primary IPT on the risk of developing isoniazid-resistant TB.

Methods

Identification and Selection of Studies

We searched 5 electronic databases (PubMed, Embase, Popline, National Library of Medicine Gateway, Cochrane Library) to identify studies of IPT published in English, French, or Spanish from 1951 to October 2003. Thesaurus and free-text terms were used in various combinations, depending on the requirements of each database (details available on request). We also searched by hand the reference lists in all identified publications and recent systematic reviews (*7*,*10–12*).

We reviewed the full text of all studies evaluating the effectiveness of primary IPT (given to persons with no history of TB), applying the following inclusion criteria: 1) compared incidence of TB in persons receiving isoniazid monotherapy versus those receiving no preventive therapy; 2) randomized controlled trial (RCT) or cohort study designs; and 3) results of susceptibility testing of positive

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cultures presented for both isoniazid and control groups, so the proportion of resistant strains could be ascertained in each group. We excluded studies conducted only in children (among whom microbiologic confirmation is less common), studies of secondary preventive therapy, and studies, or subgroups within studies, of persons with "recently active disease," many of whom had previously received isoniazid.

Data were extracted in duplicate by 2 investigators independently, using a standardized data-collection form. Data included study details (study population and size, design, intervention drug regimen, outcomes recorded) and quality measures (e.g., generation and concealment of allocation sequences, blinding, duration of and loss to follow-up).

Statistical Analysis

We estimated the incidence of TB caused by isoniazid-resistant strains separately for the isoniazid and control group of each study by dividing the number of persons with isoniazid-resistant TB by the total number of persons in that group. We chose the incidence of isoniazid-resistant TB in preference to the proportion of culture-positive TB cases that were isoniazid resistant because incidence better represents the impact (and risk for transmission) of resistant disease at the population level. Also, comparison of the proportion of resistant isolates between groups is complicated if the study population includes persons who have latent TB infection with an isoniazid-resistant organism. In the group receiving isoniazid, preventive therapy will decrease the number of reactivated TB cases attributable to isoniazid-susceptible strains but will have less effect on resistant strains, which will increase the proportion of resistant strains among subsequent cases of active TB. As a result, the proportion of isoniazid-resistant active TB cases will be higher in the isoniazid group than in the control group, even if isoniazid does not promote new resistance.

The analysis involved a number of assumptions, summarized in Table 1. In studies in which not all TB patients underwent resistance testing, we assumed that isolates tested were a random sample of all TB cases and multiplied the total number of TB cases by the proportion of isoniazid-resistant cases in the sample to estimate the total number of isoniazid-resistant cases. For example, if 1,000 persons were randomly assigned to isoniazid therapy, active TB developed in 50, 40 of these were tested, and 8 (20%) of 40 had isoniazid-resistant isolates, we then estimated a total of 10 (50×0.2) resistant TB cases and an incidence of isoniazid-resistant TB of 10 per 1,000 persons.

Relative risks (RR) for resistant TB in the isoniazid group compared to the control group were calculated for each study. The extra variation incurred by sampling isolates for resistance was incorporated into the 95% confidence intervals (CIs) of each RR. The RR could be written as the product of 2 ratios (the ratio of TB incidence in exposed/unexposed multiplied by the ratio of the proportion of resistant cases in the sample tested for the exposed/unexposed). Thus, the log RR could be expressed as the sum of the logs of these ratios, and the variance of the log RR could be calculated by a double application of a standard formula (details available on request). When no resistant cases were found in 1 of the 2 groups, we added 0.5 to the numerator and denominator of both groups when estimating the risk, and 0.1 to the numerators and denominators when calculating the variance of the ratio of proportions (*13*).

Tests of between-study heterogeneity were performed, and meta-analyses were carried out to derive summary RRs, by using a random-effects model when evidence of heterogeneity was found (*14*). In the meta-analysis, we first considered all studies as a single group, then considered separately studies from the pre-HIV era and studies of HIV-infected persons; we hypothesized that HIV-infected

Table 1. Assumptions underlying the anaylsis

Assumption	Comment
When a sample of culture positive isolates underwent resistance testing, this was a random sample of all cases.	Additional variation incurred by sampling tuberculosis (TB) cases for resistance was incorporated into 95% confidence interval estimates and thus the weighting of studies in meta-analyses.
	Differential ascertainment of resistance is unlikely because most of included studies were double-blinded and (for studies in which information was available) similar proportions of culture-positive TB cases from each group were tested.
Latent infection with isoniazid-resistant TB was equally distributed between comparison groups.	12 of 13 studies were comparisons of randomized groups; any latent infection with a resistant organism would likely be equally distributed between comparison groups. Any imbalance due to random error would be bidirectional and so would result in summary estimate of relative risk tending towards 1 (i.e., being underestimated).
Risk for isoniazid-resistant TB resulting from recent infection was equally distributed between comparison groups.	Similarly, any new infection with an isoniazid-resistant organism would likely be equally distributed between randomized groups. Any imbalance would similarly result in summary estimate of relative risk being underestimated.

persons could be at higher risk of having resistance develop. When latent TB infection is treated, few organisms are exposed to the drug (*15*). The risk for selection pressure favoring a drug-resistant organism is therefore low (*16*) unless persons have undiagnosed active TB and thus inadvertently receive monotherapy for active disease. Active TB may be more difficult to detect among HIV-infected persons, which could lead to a higher risk for undiagnosed active disease.

Sensitivity analyses primarily consisted of excluding from meta-analyses studies a) that had zero resistant cases in a group and b) that were not RCTs. Publication bias was investigated by using funnel plots and adjusted rank correlation tests (*17*). All analyses were carried out in Stata version 8.0 (Stata Corp., College Station, TX, USA).

Results

We identified 19 studies comparing primary IPT with no treatment that reported isoniazid resistance among adults (*9*,*18*–*35*). Of the 11 studies from the pre-HIV era, 4 (*23*–*26*) were excluded because resistance data from the control group were incomplete or not reported. In 2 studies (*9*,*19*), we excluded subgroups of persons with previously active disease, for which many had received isoniazid. Of the 8 studies among HIV-infected persons, 2 (*28*,*29*) were excluded because the total number of isolates tested in the relevant groups could not be determined. For 1 study (*33*), unpublished resistance data were obtained from the authors (P. Godfrey-Faussett, pers. comm.).

Characteristics of Included Studies

Thirteen studies were included in the analysis (Tables 2, 3), 12 RCTs and 1 retrospective cohort study. The 7 pre-HIV era studies (N = 32,179) were mostly conducted in the late 1950s or early 1960s in populations of persons with radiologically-inactive TB lesions (*9*,*19*,*22*), persons in communities with high TB incidence (*20*,*21*), and household contacts of TB cases (*18*); 1 study was of persons with silicosis in Hong Kong in the 1980s (*27*). Study population size ranged from 225 to 15,751 patients. In most studies, isoniazid 300 mg (or 5 mg/kg) was given daily, although in the Greenland study (*20*), 400 mg was given on 2 consecutive days each week. Duration of treatment was 24 weeks to 2 years. All 6 studies among HIV-infected persons (N = 3,901) recruited participants from HIV clinics or voluntary counseling and testing centers. Study population size was 121–1,718. RCTs administered isoniazid for 6 months at 300 mg daily (*30*,*31*,*34*,*35*) or 900 mg twice weekly (*33*); in the cohort study, an unspecified dose was given for 9 to 12 months (*32*).

We could assess the method of assigning the treatment allocation in 5 of the 12 RCTs: 2 studies (*31*,*33*) used computer-generated random numbers, 2 (*20*,*21*) used random number tables, and 1 (*19*) assigned by odd or even hospital number. Three RCTs reported that the treatment was concealed: 2 used sealed envelopes (*33*,*34*), and 1 used numbered packages containing isoniazid or matching placebo (*27*). Eight RCTs were double-blinded (*18*,*20*,*21*,*27*,*30*,*31*,*33*,*34*), although in 1 study, isoniazid and placebo groups may have received different numbers of tablets (*34*); 2 were not blinded (*19*,*35*), and 2 did not report blinding (*9*,*22*). Loss to follow-up was reported in 11 studies: in 6, this loss was <20% in both groups (see unabridged, online versions of Tables 2 and 3, available at http://www.cdc.gov/ncidod/eid/vol12no05/05-0681.htm#table2).

Tuberculosis Cases and the Proportion of Isoniazid-resistant Isolates

The total number of TB cases within a study ranged from 7 to 561. In all studies combined, 564 TB cases occurred among persons who received isoniazid, and 1,034 occurred among controls. In the 7 studies that reported this information, 55%–100% of TB cases were sputum-culture positive (*20*,*22*,*27*,*31*,*32*,*34*,*35*). In 4 of these studies, ≥90% of culture-positive isolates underwent resistance testing (*22*,*27*,*31*,*35*). In total, 158 persons in the isoniazid groups and 328 in control groups had isolates tested for resistance to isoniazid. Definitions of isoniazid resistance varied, and the proportion of tested isolates that were resistant ranged from 0% to 100% (Tables 2, 3, and unabridged online versions). A total of 31 resistant isolates were obtained from the isoniazid groups and 28 or 24 (depending on the definition of resistance) from control groups. Of the 6 studies among HIV-infected persons, 1 found no resistant isolates in the isoniazid group (*33*), 2 found no resistant isolates in the control group (*31*,*32*), and 1 found no resistance in either group (*30*) (Table 3).

Relative Risk for Isoniazid Resistance and Meta-analyses

In 8 of the 12 studies in which a single definition of resistance was used, the point estimate of RR for isoniazid resistance in the isoniazid group compared to that of controls was >1, although this result was not statistically significant in any study (Tables 2, 3). Two alternative (and substantially different) definitions of resistance were used in the Greenland study, which resulted in different estimates of the effect of IPT on isoniazid resistance (Table 2). We therefore conducted 2 analyses, using each definition of resistance for this study. By using definition (a) from the Greenland study, the summary RR for all 13 studies combined was 1.25 (95% CI 0.75–2.10) in either a random or fixed effects model (Figure 1A) with little evidence of heterogeneity (P_{het} = 0.789). By using definition (b) from the Greenland study, the summary RR was 1.45 (95% CI

Table 2. Studies comparing isoniazid treatment with no treatment in HIV-uninfected populations*

Author, country, dates	Population	Intervention/ comparison; blinding	Enrolled (n) INH/control	TB cases: culture positive/total (%)		Resistant cases/total tested (% culture positive tested)		Risk for resistant TB/1,000		RR (95% CI)
				INH	Controls	INH	Controls	INH	Controls	
Ferebee, USA, 1957–NS (*18*)	Household contacts of TB patients	12 mo INH, 4–7 mg/kg/day/placebo; double blind	7,755/7,996	NS/86	NS/215	2/10 NS	2/31 NS	2.22	1.73	1.28 (0.20–8.07)
Katz, USA, 1958–1964 (*19*)	Mental hospital patients with inactive lesions	2 y INH, 300 mg daily/no treatment; not blind	118/107	NS/9	NS/10	1/1 NS	2/5 NS	76.27	37.38	2.04 (0.52–8.08)
Horwitz, Greenland, 1956–1963 (*20*)	76 villages	2 × 13 wk INH, 400 mg twice weekly/0.1 mg INH; double blind	4,174/3,907	123/238 (51.7)	186/323 (57.6)	(a) 2/46†	(a) 5/66†	(a) 2.48†	(a) 6.26†	(a) 0.40 (0.08–1.97)†
						(b) 2/46 (37)‡	(b) 1/66 (36)‡	(b) 2.48‡	(b) 1.25‡	(b) 1.98 (0.18–21.31) ‡
Comstock, USA (Alaska), 1957–1964 (*21*)	Residents of 28 villages and 2 boarding schools	12 mo INH, 300 mg§ daily/ placebo; double blind	3,047/3,017	NS/58	NS/141	4/20 NS	1/50 NS	3.81	0.93	4.07 (0.47–34.98)
Ferebee, USA, 1960–1967 (*9*)	Persons with inactive lesions	12 mo INH, 5 mg/kg/day/ placebo; NS	701/714	NS/18	NS/49	2/5 NS	2/25 NS	10.27	5.49	1.87 (0.31–11.19)
Pamra, India, 1958–1968 (*22*)	X-ray screening attendees with inactive TB	12 mo INH, 3–4 mg/kg/day/ placebo; NS	139/178	10/18 (55.6)	57/76 (75)	3/9 (90)	6/52 (91)	43.17	49.27	0.88 (0.24–3.15)
Hong Kong Chest Service, Hong Kong, 1981–1987 (*27*)	Men with silicosis	24 wk INH, 300 mg daily/placebo; double blind	167/159	19/25 (76)	29/36 (80.6)	5/19 (100)	4/28 (97)	39.39	32.35	1.22 (0.34–4.32)

*INH, isoniazid; TB, tuberculosis; RR, relative risk; CI, confidence interval; NS, not stated; med., median; Rx, treatment. Because of space limitations, some data have been removed; see online version for complete table.
†(a), definition of resistance as ≥1 colony growth at ≥0.32 μg/mL INH.
‡(b), definition of resistance as growth equal to control tube at ≥0.32 μg/mL INH.
§Children were given 5 mg/kg/day INH.

0.85–2.47, Figure 1B), again with little evidence of heterogeneity (P_{het} = 0.923). Summary estimates were virtually unaltered when analyses were restricted to RCTs without zero cells (Figure 1). We also excluded the Greenland study from the meta-analysis to assess its overall effect on the summary estimate. The summary RR using the remaining 12 studies was similar to that obtained by using definition (b) for resistance (RR 1.43, 95% CI 0.83–2.46).

Among the 7 studies from the pre-HIV era, the summary RR for isoniazid resistance was 1.24 (95% CI 0.69–2.21) when the definition (a) from the Greenland study was used and 1.50 (95% CI 0.82–2.73) with definition (b). The summary RR was 1.30 (95% CI 0.42–4.02) for the 6 studies of HIV-infected persons. Little evidence of between-study heterogeneity was found in any of these analyses (P_{het} >0.5 for all). When meta-analysis of the studies of HIV-infected persons was restricted to the 2 RCTs without zero cells (*34*,*35*), the summary RR rose slightly to 1.42 (95% CI 0.26–7.69) in a random-effects model, with slightly stronger evidence of heterogeneity (P_{het} = 0.179). Funnel plots (Figure 2) suggested little evidence of publication bias (p = 0.625 and p = 0.542 by using definition [a] and definition [b], respectively, for the Greenland study).

Table 3. Studies comparing isoniazid treatment with no treatment in HIV-infected populations*

Author, country, dates	Population	Intervention/ comparison; blinding	Enrolled (n) INH/control	TB cases: culture positive/total (%)		Resistant cases/total tested (% culture positive tested)		Risk for resistant TB/1,000		RR (95% CI)
				INH	Controls	INH	Controls	INH	Controls	
Randomized controlled trials										
Gordin, USA, 1991–1996 (*30*)	Clinic attendees; med. CD4 233/247	6 mo INH 300 mg daily vs. placebo; double blind	260/257	NS/3	NS/6	0/3 (NS)	0/5 (NS)	1.92†	1.94†	0.99 (0.06–6,298.19)
Hawken, Kenya, 1992–1997 (*31*)	Clinic or VCT attendees; med. CD4 321.5/346	6 mo INH 300 mg daily/placebo; double blind	342/342	19/25 (76)	22/23 (95.7)	2/17 (90)	0/21 (96)	10.05†	1.46†	6.88 (0.01–3,882.85)
Mwinga, Zambia, 1992–1996 (*33*)	VCT attendees	6 mo INH 900 mg twice weekly/placebo; double blind	350/352	NS/27	NS/44	0/3 (NS)	1/5 (NS)	1.43†	26.38†	0.05 (0.00–30.47)
Johnson, Uganda, 1993–NS (*34*)	Clinic or counseling attendees	6 mo INH 300 mg daily/placebo; partially double blind‡	931/787	36/51 (70.6)	46/64 (71.9)	5/20 (56)	1/24 (52)	13.69	3.39	4.04 (0.50–32.80)
Rivero, Spain, 1994–2000 (*35*)	Clinic attendees; med. CD4 193/215	6 mo INH 300 mg daily/no treatment; not blind	82/77	3/3 (100)	4/4 (100)	3/3 (100)	4/4 (100)	36.59	51.95	0.70 (0.16–3.05)
Cohort study										
Moreno, Spain, 1985–1994 (*32*)	Clinic attendees; med. CD4 689/648	9–12 mo INH (dose NS)/no treatment; not blind	29/92	3/3 (100)	39/43 (90.7)	2/2 (67)	0/12 (31)	118.64†	5.41†	21.95 (0.04–11,582.31)

*INH, isoniazid; TB, tuberculosis; RR, relative risk; CI, confidence interval; med., median; NS, not stated; Rx, treatment; VCT, voluntary counseling and testing; PPD, purified protein derivative. Because of space limitations, some data have been removed; see online version for complete table.
†Calculated by adding 0.5 to numerator and denominator of both groups.
‡Unclear whether isoniazid and placebo group received the same number of tablets.

Discussion

Our summary RR for isoniazid-resistant TB after IPT is not statistically significant, but the point estimate and upper boundary of the 95% CI are consistent with an increased risk. Our review highlights the limitations of existing data; however, since further individually randomized, controlled trials of IPT would be inappropriate, additional data of this type are unlikely to become available.

The numbers of TB cases in the individual studies were often small, and in 4 studies, no resistant TB cases occurred in at least 1 of the comparison groups. Comparison of summary estimates with and without these 4 studies suggests that adding a small number to the numerators and denominators so they could contribute to summary estimates did not in itself affect the result. The 95% CIs for RRs in these studies were very wide, and so their contribution to the summary RR estimate was limited.

The summary estimate of effect was similar in HIV-infected and HIV-uninfected persons. Screening for active TB before enrollment could have been more rigorous in studies among HIV-infected persons; the screening procedures were not always clearly described.

The proportion of positive cultures tested for resistance varied from 37% to 100%; why all isolates were not tested was not clear. The most important assumption made in the analysis was that the proportion of resistant cases among the isolates tested was representative of all TB cases in that group. If investigators were not blinded to the treatment allocation, and if persons receiving isoniazid were more likely to have positive cultures tested for resistance, ascertainment of resistance in the isoniazid group could have increased, and thus RR could have been overestimated. However, in 10 of the 13 studies, a placebo was used; 8 studies specified that the trial was double blinded, and (for studies for which information was available) similar proportions of culture-positive TB cases were tested from each group. Therefore, differential ascertainment of resistance is unlikely. Our estimate of the total number of isoniazid-resistant cases disregarded whether case-patients

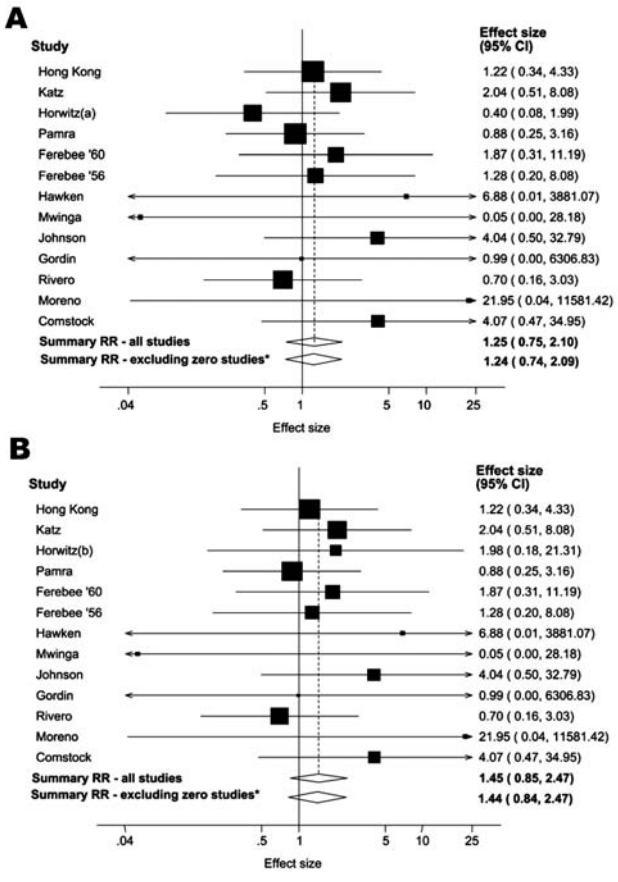

Figure 1. Relative risk (RR) for isoniazid resistance associated with isoniazid preventive therapy in 13 studies. A) Using definition (a) of resistance for the Greenland study (*20*). B) Using definition (b) of resistance for the Greenland study. * Excluding the 4 studies with no resistant cases in 1 or both of the 2 groups. The squares and horizontal lines represent the relative risk (RR) and 95% confidence intervals (CIs) for each study. The diamonds represent the summary RR and 95% CIs.

were sputum-culture positive. Persons with isoniazid-resistant isolates that are sputum-culture negative are less likely to transmit disease and present less of a public health concern. This situation is unlikely to affect our estimate of the effect of isoniazid on the incidence of resistant disease, but our estimate may exaggerate the public health risk.

Study quality and review methods may have affected the results in other ways. For example, inadequate random assignment of HIV-infected persons could result in more advanced immunosuppression among those in the isoniazid group and thus a higher probability of resistance. However, when reported, the method of randomization in trials of HIV-infected persons appeared adequate. Differences in loss to follow-up between comparison groups could affect results if those who were lost to follow-up had a different probability of resistance than those not lost. In 6 of the 11 RCTs with information, <20% were lost to follow-up in both groups, but the loss was noticeably higher in the isoniazid group than for controls in 2 studies of HIV-infected persons (*34*,*35*). Publication bias could affect the results if studies finding increased resistance among persons receiving isoniazid were more likely to be published. However, the aim of all the studies was to investigate effectiveness of IPT, not to ascertain development of resistance, and our analyses suggest that publication bias did not affect the summary estimate.

The methods used to test for isoniazid resistance are now relatively standardized and based on the proportion method in which resistance is defined as growth on medium containing 0.2 μg/mL isoniazid that exceeds 1% of the growth on control medium (*36*). In older studies, methods were less standardized and were based on absolute numbers of colonies growing on media with various concentrations of antituberculous drugs. In the Greenland study,

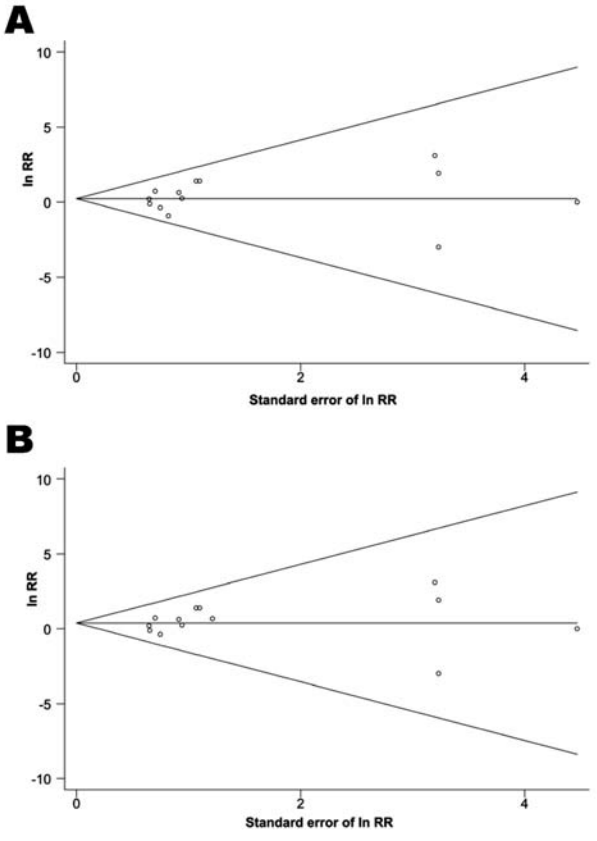

Figure 2. Funnel plots to detect publication bias for studies reporting the effect of isoniazid preventive therapy on risk for isoniazid-resistant tuberculosis. The log relative risk (RR) for each study is plotted against the standard error of the natural log (ln) of the RR. The horizontal line indicates the (log) summary RR, and guidelines to assist in visualizing the funnel are plotted at the 95% pseudo-confidence limits about the summary RR estimate. A) Using definition (a) of resistance for the Greenland study (*20*); B) using definition (b) of resistance for the Greenland study.

results for resistance were presented by using 2 divergent definitions (neither corresponding to modern methods), and these gave quite different estimates of effect. Definition (a) is likely to have led to an overestimation of resistance in both groups; definition (b) is likely to have led to an underestimation of resistance in both groups. When this study was excluded from the analysis, the summary estimate was similar to that using definition (b), which suggests that the estimates using definition (a) were more anomalous.

Studies using DNA fingerprinting illustrate that in settings with a high prevalence of TB, newly acquired infection is an important cause of active TB (*37*,*38*). Thus, isoniazid-resistant TB may be newly acquired rather than attributable to any previous IPT. However, any such effect should be equally distributed between randomized groups (Table 1).

IPT is a safe, low-cost intervention that has the potential to reduce illness and death caused by TB, especially among HIV-infected persons. The main cause of antituberculous drug resistance is inadequate treatment of active TB. Therefore, any risk for a small increase in the incidence of isoniazid resistance attributable to wider use of IPT needs to be weighed against its benefit in reducing TB incidence.

If IPT does increase the risk for isoniazid-resistant TB, one might argue that combination regimens should be used. Combination regimens have similar efficacy to isoniazid alone among HIV-infected persons and are shorter, but these regimens generally have more adverse effects (*7*,*39*), are more expensive, and risk promoting resistance to rifampin. We did not compare the risk for antituberculous drug resistance with IPT versus combination regimens.

Our review highlights the paucity of available data and does not exclude an increased risk for isoniazid-resistant TB after IPT. IPT substantially reduces the risk for active TB disease in persons whose tuberculin skin test is positive, and we support the expansion of its use, in line with recent recommendations from the HIV/TB working group of the Stop TB partnership (*40*). If the main reason for the development of resistance among persons receiving IPT is failure to diagnose active TB, our results underscore the need for effective diagnostic strategies and tests. In accordance with WHO policy, ongoing surveillance for isoniazid resistance is required among populations in which this intervention is widely implemented.

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etymologia

tuberculosis

[too-ber″ku-lo′sis]

Any of the infectious diseases of humans or other animals caused by bacteria of the genus *Mycobacterium*. From the Latin *tuberculum*, "small swelling," the diminutive form of tuber, "lump." Tuberculosis has existed in humans since antiquity; it is believed to have originated with the first domestication of cattle. Evidence of tuberculosis has been shown in human skeletal remains and mummies from as early as 4000 BC. *Mycobacterium bovis* bacillus Calmette-Guérin has been successfully used to immunize humans since 1921, and treatment (rather than prevention) of tuberculosis has been possible since the introduction of streptomycin in 1946. Hopes of completely eliminating the disease, however, have been diminished since the rise of drug-resistant *M. tuberculosis* strains in the 1980s.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; Merriam-Webster's collegiate dictionary. 11th ed. Springfield (MA): Merriam-Webster Incorporated; 2003; and wikipedia.org

Mycobacterium tuberculosis and Rifampin Resistance, United Kingdom

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The United Kingdom Health Protection Agency Mycobacterium Reference Unit offers a national "Fastrack" molecular service for detecting *Mycobacterium tuberculosis* complex (MTBC) and rifampin resistance by using the INNO-LiPA Rif.TB assay. We analyzed the service in a routine, nontrial context of 1,997 primary clinical specimens, including 658 nonrespiratory specimens. The overall adjusted concordance, sensitivity, specificity, positive predictive value, and negative predictive value for detecting MTBC were 91.2%, 85.2%, 96.2%, 95.7%, and 86.7%, respectively (unadjusted, 86.7%, 85.2%, 88.2%, 86.9%, and 86.7%), when false-positive samples from patients (n = 83) with a known microbiologic diagnosis of MTBC or patients receiving current or recent antituberculous treatment were excluded. The parameters for detecting rifampin resistance were 99.1%, 95.0%, 99.6%, 92.7%, and 99.7%, respectively. The assay enabled earlier diagnosis of MTBC and rifampin resistance (15.2 days) compared with culture-based techniques (30.7 days).

The increasing incidence of multidrug-resistant tuberculosis (MDRTB), defined as resistance to at least rifampin and isoniazid, is a notable global health problem (*1*). The rapid identification of patients with MDRTB enables early institution of appropriate treatment, which is associated with improved survival (*2*,*3*), and infection control procedures to minimize risk of transmission (*4*). The Centers for Disease Control and Prevention recommends that the culture/identification and susceptibility testing of *Mycobacterium tuberculosis* complex (MTBC) be completed within 21 and 30 days of specimen receipt, respectively (*5*). Molecular assays based on the genetics of drug resistance may considerably reduce these turnaround times.

In the United Kingdom, 82.5% of rifampin-resistant isolates are also resistant to isoniazid (*6*), making rifampin resistance a useful surrogate marker for MDRTB. Most rifampin-resistant MTBC strains have mutations in an 81-bp region of the *rpoB* gene that encodes the RNA polymerase β subunit (*7*). This region is therefore an ideal target for molecular tests for rifampin resistance.

The United Kingdom Health Protection Agency Mycobacterium Reference Unit (MRU) offers a national molecular diagnostic service (Fastrack) for detection of MTBC and rifampin resistance (*8*) by using the INNO-LiPA Rif.TB assay (Innogenetics, Zwijndrecht, Belgium) and supplemented by DNA sequencing as needed. This assay is based on reverse hybridization between *rpoB* amplicons and membrane-bound capture probes (1 specific for MTBC, 5 overlapping wild-type probes spanning the *rpoB* target region, and 4 of the most common mutations). Genotypic resistance is indicated by absence of hybridization with wild-type probes or hybridization with resistance mutation probes (*9*).

A review of the line probe assay (LiPA) (*10*) found that most previous evaluations focused on mycobacterial isolates and culture-positive (mainly respiratory) specimens (*9*,*11*–*14*), but relatively little data exist on nonrespiratory and smear-negative specimens, which are often collected in routine clinical practice (*8*,*15*,*16*). The Fastrack service was initially targeted at smear-positive respiratory samples and mycobacterial isolates, but in response to widespread demand from other laboratories, was extended to all specimens, regardless of acid-fast bacilli (AFB) status. In January 2002, an in-house polymerase chain reaction (PCR) assay targeting the IS*6110* insertion sequence (*17*) replaced LiPA for testing cerebrospinal fluid (CSF) samples. Therefore, CSF samples were not included in this study. This study evaluated LiPA in the context of a nontrial clinical service in one of the largest reported samples of

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1,997 primary clinical specimens (including 658 nonrespiratory) and 290 clinical isolates tested from 1999 to 2002.

Materials and Methods

Clinical Specimens

From January 1999 through December 2002, the MRU received 2,287 consecutive non-CSF specimens from 2,110 patients (comprising 1,997 primary clinical specimens and 290 clinical isolates) from 152 centers in the United Kingdom and Ireland for Fastrack analysis. Specimens are submitted for analysis at the discretion of individual referring laboratories, usually when the diagnosis of MTBC is uncertain or when rifampin resistance is suspected. When multiple specimens were received from a single patient, each specimen was processed separately. Of the primary specimens, 1,339 respiratory specimens were sputum, bronchial washings, and bronchial and tracheal aspirates; 658 were nonrespiratory specimens. Samples were received only on weekdays, and routine processing and culture were initiated within 24 hours of receipt. Turnaround times for completion of analysis, culture, and identification of MTBC and drug-susceptibility testing were calculated from date of specimen receipt (*5*).

Routine Microscopy, Culture, Identification, and Susceptibility Testing

Samples were decontaminated by using the NaOH/N-acetyl-L-cysteine method in a 2-mL suspension, and AFB staining was performed with auramine-phenol and the Ziehl-Neelsen procedure (*18*,*19*). DNA was extracted from 1 mL of decontaminated specimen by using a previously described chloroform extraction technique (*20*), and the remaining 1 mL was added to 1 MB/BacT rapid culture vial (bioMérieux UK Ltd., Basingstoke, UK) and 1 Lowenstein-Jensen slope. Cultures were incubated for at least 8 weeks. Mycobacterial cultures were identified by microscopic and macroscopic appearances, biochemical tests, and DNA hybridization with Accuprobe (GenProbe, San Diego, CA, USA). Drug-susceptibility testing was carried out by the resistance ratio method (*18*).

LiPA

LiPA was performed according to manufacturer's instructions. The first round of a nested PCR was performed with 10 μL of DNA extract and outer primers (LiPA OP1, 5′-GAGAATTCGGTCGGCGAGCTGATCC-3′ and LiPA OP2, 5′-CGAAGCTTGACCCGCGCGTACACC-3′) for 30 cycles at 95°C for 60 s, 58ºC for 30 s, and 72ºC for 90 s. One microliter of first-round product was transferred to a 40-μL PCR mixture containing inner primers (LiPA IP1, 5′-GGTCGGCATGTCGCGGATGG-3′ and LiPA IP2, 5′-GCACGTCGCGGACCTCCAGC-3′), which were biotinylated at the 5′ end, for the second round of amplification for 30 cycles at 95°C for 20 s, 65ºC for 30 s, and 72°C for 30 s. Each PCR run included a duplicate and an inhibition control (100 genome copies of *Mycobacterium bovis* bacillus Calmette-Guérin [BCG]) for each sample, 5 extracted, water, negative controls, decontaminated, extracted, negative and positive controls (a known culture-positive clinical sample), and a positive control with a low amount of DNA (10 genome copies of BCG in 10 μL). A 260-bp band on agarose gel electrophoresis confirmed successful amplification. The hybridization assay to determine genotypic rifampin resistance was then performed and analyzed as previously described (*13*). The MTBC result was then reported as positive (accompanied by a rifampin-susceptibility result), negative, equivocal, or inhibited. Results were considered equivocal if a sample tested PCR positive on 1 of 2 duplicates on 2 separate occasions. Extracted DNA was stored for retesting equivocal and inhibited results and for future resolution of discrepant susceptibility results.

Sequencing of *rpoB* PCR Product

Cultures of MTBC with discordant rifampin-susceptibility results by phenotypic and LiPA testing underwent automated sequencing of the *rpoB* PCR products with either the Long Read Tower System (Visible Genetics, Suwanee, GA, USA) or the CEQ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK). DNA was extracted from cultures and amplified in a PCR containing the outer primers OP1 and OP2 and sequenced with the inner primers IP1 and IP2.

Statistical Analysis

Data were entered into Microsoft Access (Microsoft Corp., Redmond, WA, USA) and analyzed with Microsoft Excel. Detection of MTBC and rifampin resistance by LiPA was compared with results by the accepted standards of culture and phenotypic susceptibility testing. Concordance, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. We excluded 85 (3.7%) samples from primary analysis because LiPA results could not be compared with culture results. These samples had equivocal PCR results (n = 27, 1.2%), were inhibitory to PCR (n = 22, 1.0%), could not be cultured (e.g., because of insufficient volume or histologic samples embedded in paraffin wax; n = 6, 0.3%), or were contaminated with bacteria or fungi (n = 30, 1.3%).

Results

Microscopy and Culture

Of the primary specimens tested by LiPA, the AFB smear microscopy was positive in 1,137 (56.9%), negative

in 821 (41.1%), and not performed in 39 (2.0%). Specimen types are shown in Tables 1 and 2. Culture identification and drug susceptibility results are shown in Table 3. MTBC was cultured from 941 (47.1%) of 1,997 primary samples and 238 (82.1%) of 290 isolates. In 3 cases, both MTBC and nontuberculous mycobacteria were cultured. A total of 1,178 *M. tuberculosis*, 10 *M. bovis* (including 1 BCG), and 1 *M. africanum* cultures were identified in the

Table 1. Results of LiPA compared with culture in detecting MTBC in primary clinical specimens*

Sample and AFB smear result	No. positive/no. tested (%)					Mean days saved
	Concordance	Sensitivity	Specificity	PPV	NPV	
All primary LiPA	1,667/1,922 (86.7)	782/918 (85.2)	886/1,004 (88.2)	782/900 (86.9)	886/1,022 (86.7)	15.2
Positive	960/1,099 (87.4)	747/798 (93.6)	213/301 (70.8)	747/835 (89.5)	213/264 (80.7)	14.8
Negative	679/792 (85.7)	35/119 (29.4)	644/673 (95.7)	35/64 (54.7)	644/728 (88.5)	22.1
Not done	28/31 (90.3)	0/1 (0)	29/30 (96.7)	0/1 (0)	29/30 (96.7)	–
All primary LiPA (adjusted values)†	1,667/1,828 (91.2)	782/918 (85.2)	886/921 (96.2)	782/817 (95.7)	886/1,022 (86.7)	15.2
Positive	960/1,028 (93.4)	747/798 (93.6)	213/232 (91.8)	747/766 (97.5)	213/264 (80.7)	14.8
Negative	679/771 (88.1)	35/119 (29.4)	644/659 (97.7)	35/50 (70.0)	644/728 (88.5)	22.1
Not done	28/29 (96.6)	0/1 (0)	29/30 (96.7)	0/1 (0)	29/30 (96.7)	–
Respiratory	1,168/1,298 (90.0)	672/738 (91.1)	496/560 (88.6)	672/736 (91.3)	496/562 (88.3)	14.7
Positive	827/915 (90.4)	657/696 (94.4)	170/219 (77.6)	657/706 (93.1)	170/209 (81.3)	14.5
Negative	328/369 (88.9)	15/42 (35.7)	313/327 (95.7)	15/29 (51.7)	313/340 (92.1)	20.5
Not done	13/14 (92.9)	–	13/14 (92.9)	0/1 (0.0)	13/13 (100.0)	–
Nonrespiratory	499/624 (80.0)	110/180 (61.1)	390/444 (87.8)	110/164 (67.1)	390/960 (84.8)	18.3
Positive	133/184 (72.3)	90/102 (88.2)	43/82 (52.4)	90/129 (69.8)	43/55 (78.2)	17.2
Negative	351/423 (83.0)	20/77 (26.0)	331/346 (95.7)	20/35 (57.1)	331/388 (85.3)	23.4
Not done	15/17 (88.2)	0/1 (0)	16/16 (100.0)	–	16/17 (94.1)	––
Biopsy specimen‡	92/108 (85.2)	13/26 (50.0)	79/82 (96.3)	13/16 (81.3)	79/92 (85.9)	22.4
Positive	19/21 (90.5)	10/10 (100.0)	9/11 (81.8)	10/12 (83.3)	9/9 (100.0)	24.1
Negative	72/86 (83.7)	3/16 (18.8)	69/70 (98.6)	3/4 (75.0)	69/82 (84.1)	16.7
Not done	1/1 (100.0)	–	1/4 (25.0)	–	1/1 (100.0)	–
Gastric aspirate	17/18 (94.4)	4/5 (80.0)	13/13 (100.0)	4/4 (100.0)	13/14 (92.9)	16.8
Positive	8/8 (100.0)	4/4 (100.0)	4/4 (100.0)	4/4 (100.0)	4/4 (100.0)	16.8
Negative	9/10 (90.0)	0/1 (0.0)	9/9 (100.0)	–	9/10 (90.0)	–
Not done	–	–	–	–	–	–
Lymph node	103/142 (72.5)	50/68 (73.5)	53/74 (71.6)	50/71 (70.4)	53/71 (74.6)	18.7
Positive	54/79 (68.4)	42/48 (87.5)	12/31 (38.7)	42/61 (68.9)	12/18 (66.7)	16.6
Negative	48/62 (77.4)	8/20 (40.0)	40/42 (95.2)	8/10 (80.0)	40/52 (76.9)	29.6
Not done	1/1 (100.0)	–	1/1 (100.0)	–	1/1 (100.0)	–
Pleural fluid	84/107 (78.5)	5/23 (21.7)	79/84 (94.0)	5/10 (50.0)	79/97 (81.4)	26.8
Positive	6/10 (60.0)	4/5 (80.0)	2/5 (40.0)	4/7 (57.1)	2/3 (66.7)	23.5
Negative	77/97 (79.4)	1/18 (5.6)	76/78 (97.4)	1/3 (33.3)	76/93 (81.7)	40.0
Not done	1/1 (100.0)	–	1/1 (100.0)	–	1/1 (100.0)	–
Psoas abscess	8/15 (53.3)	3/6 (50.0)	5/9 (55.6)	3/7 (42.9)	5/8 (62.5)	19.0
Positive	2/3 (66.7)	2/2 (100.0)	0/1 (0.0)	2/3 (66.7)	–	17.0
Negative	6/12 (50.0)	1/4 (25.0)	5/8 (62.5)	1/4 (25.0)	5/8 (62.5)	23.0
Not done	–	–	–	–	–	–
Vertebral aspirate	26/30 (86.7)	10/12 (83.3)	16/18 (88.9)	10/12 (83.3)	16/18 (88.9)	15.4
Positive	9/10 (90.0)	8/8 (100.0)	1/2 (50.0)	8/9 (88.9)	1/1 (100.0)	14.5
Negative	17/19 (89.5)	2/3 (66.7)	15/16 (93.8)	2/3 (66.7)	15/16 (93.8)	19.0
Not done	0/1 (0.0)	0/1 (0.0)	–	–	0/1 (0.0)	–
Other§	169/204 (82.8)	25/40 (62.5)	145/164 (88.4)	25/44 (56.8)	145/160 (90.6)	15.2
Positive	35/53 (66.0)	20/25 (80.0)	15/28 (53.6)	20/33 (60.6)	15/20 (75.0)	15.1
Negative	122/138 (88.4)	5/15 (33.3)	117/123 (95.1)	5/11 (45.5)	117/127 (92.1)	15.8
Not done	12/13 (92.3)	–	13/13 (100.0)	–	13/13 (100.0)	–

*MTBC excludes 75 specimens containing substances inhibitory to the polymerase chain reaction (PCR), PCR-equivocal results, and samples with no definitive culture results (i.e., contaminated or not done). LiPA, line probe assay; MTBC, *Mycobacterium tuberculosis* complex; AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value.
†Excludes samples from patients with a microbiologic diagnosis of MTBC made at the Mycobacterium Reference Unit in the last 18 or subsequent 3 months, and patients receiving antituberculous treatment currently or within the last 3 months.
‡Includes biopsy specimens from liver (n = 13), kidney (n = 2), skin (n = 15), lung (n = 20), pleura (n = 13), and miscellaneous sites (n = 45).
§Includes ascites (n = 56), pericardial aspirates (n = 29), aspirates from miscellaneous sites (n = 80), blood (n = 1), bone marrow (n = 22), feces (n = 1), and urine (n = 15).

Table 2. Results of LiPA in detecting rifampin resistance in specimens from which MTBC was correctly identified and cultured*

Sample and AFB smear result	No. positive/no. tested (%)					Mean days saved
	Concordance	Sensitivity	Specificity	PPV	NPV	
All primary LiPA	775/782 (99.1)	38/40 (95.0)	737/740 (99.6)	38/41 (92.7)	738/740 (99.7)	30.7
Positive	743/747 (99.5)	35/36 (97.2)	708/710 (99.7)	35/37 (94.6)	709/710 (99.9)	30.4
Negative	32/35 (91.4)	3/4 (75.0)	29/30 (96.7)	3/4 (75.0)	29/30 (96.7)	37.2
Not done	–	–	–	–	–	–
Respiratory	669/672 (99.6)	32/33 (97.0)	637/639 (99.7)	32/34 (94.1)	637/638 (99.8)	30.4
Positive	654/657 (99.5)	31/32 (96.9)	623/625 (99.7)	31/33 (93.9)	623/624 (99.8)	30.2
Negative	15/15 (100.0)	1/1 (100.0)	14/14 (100.0)	1/1 (100.0)	14/14 (100.0)	39.1
Not done	–	–	–	–	–	–
Nonrespiratory	106/110 (96.4)	6/7 (85.7)	100/101 (99.0)	6/7 (85.7)	101/102 (99.0)	32.5
Positive	89/90 (98.9)	4/4 (100.0)	85/85 (100.0)	4/4 (100.0)	86/86 (100.0)	32.0
Negative	17/20 (85.0)	2/3 (66.7)	15/16 (93.8)	2/3 (66.7)	15/16 (93.8)	35.5
Not done	–	–	–	–	–	–
Clinical isolate	229/235 (97.4)	21/23 (91.3)	208/211 (98.6)	21/24 (87.5)	208/210 (99.0)	16.3

*LiPA, line probe assay; MTBC, *Mycobacterium tuberculosis* complex; AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value.

4-year study period. During this time, 6,500–7,000 cases of tuberculosis were reported in the United Kingdom per year, including 4,500–5,000 reported to be culture positive (*6*). The times taken to culture MTBC from primary specimens are shown in Table 4. There were 223 nontuberculous mycobacteria isolates: 80 *M. avium* complex, 38 *M. kansasii*, 26 *M. xenopi*, 23 *M. malmoense*, 20 *M. chelonae*, 10 *M. fortuitum*, 3 *M. abscessus*, 3 *M. marinum*, 2 *M. gordonae*, 2 *M. simiae*, 2 *M. terrae*, 1 *M. szulgai*, 1 *M. vaccae*, and 12 unidentified *Mycobacterium* species.

LiPA

Results of LiPA analysis for MTBC were positive in 1,153 (50.4%), negative in 1,085 (47.4%), equivocal in 27 (1.2%), and inhibited in 22 (1.0%) primary specimens. Of the 1,153 PCR-positive samples, 1,085 (94.1%) were reported as rifampin susceptible by LiPA, and 68 (5.9%) were reported as rifampin resistant. Of the 27 PCR-equivocal samples, 16 grew MTBC (6 AFB negative, 1 AFB unknown, 9 AFB positive), 3 grew *M. avium* complex, and 8 yielded no mycobacterial growth. Tables 1 and 2, respectively, show the results of LiPA in detecting MTBC from primary specimens and rifampin resistance from specimens that grew MTBC. Data on antituberculous treatment were incomplete, but when reported, 195 (9.8%) samples were from patients receiving treatment currently or within the last 3 months. A total of 309 (15.5%) had a history of antituberculous treatment (Tables 5 and 6).

Discrepant Results

There were 136 false-negative MTBC results, i.e., samples negative by LiPA that subsequently yielded MTBC on culture. There were 118 apparently false-positive MTBC results by LiPA, which were PCR positive but did not grow MTBC, although 88 were AFB positive. A total of 83 false-positive samples were considered to have correct molecular results because they were from patients with a microbiologic diagnosis of MTBC made at MRU from another sample (n = 61) or from patients who were receiving antituberculous treatment currently or had received it within the last 3 months (n = 22). These 83 samples were excluded from statistical analysis to give adjusted values for specificity and PPV (Table 1). Ten specimens were from 6 patients with discrepant results for rifampin susceptibility (Table 7).

Table 3. Final culture identification results for all specimens*

Result	Primary specimens, no. (%)	Isolates, no. (%)	Total, no. (%)
MTBC			
Rifampin sensitive†	892 (94.9)	214 (90.9)	1,106 (93.9)
Rifampin resistant only†	9 (1.0)	3 (1.3)	12 (1.0)
MDR-TB†	37 (3.9)	20 (8.4)	57 (4.8)
Susceptibilities not determined†	2 (0.2)	1 (0.4)	3 (0.3)
Total MTBC	940 (47.1)	238 (82.1)	1,178 (51.5)
NTM	181 (9.1)	42 (14.5)	223 (9.8)
Contaminated	22 (1.1)	8 (2.8)	30‡ (1.3)
Culture not done	5 (0.3)	1 (0.3)	6‡ (0.3)
No mycobacterial spp.	849 (42.5)	1 (0.3)	850 (37.2)
Total	1,997	290	2,287

*MTBC, *Mycobacterium tuberculosis* complex; MDR-TB, multidrug-resistant tuberculosis; NTM, nontuberculous mycobacteria.
†Percentages of total MTBC cultures.
‡These 36 (1.6%) cases without definitive culture results were excluded from analyses of assay performance.

Table 4. Mean time in days to culture MTBC from all primary specimens (including those from patients receiving treatment), stratified according to smear microscopy result*

	PCR result			
AFB stain result	Positive (n)	Negative (n)	Equivocal (n)	Total (n)
Positive	18.0 (747)	23.3 (51)	22.0 (9)	18.4 (807)
Negative	25.4 (35)	31.3 (84)	30.0 (6)	29.6 (125)
Not done	0 (0)	22.0 (1)	21.0 (1)	21.5 (2)
Total	18.4 (782)	28.2 (136)	24.9 (16)	19.9 (934)

*MTBC, *Mycobacterium tuberculosis* complex; PCR, polymerase chain reaction; AFB, acid-fast bacilli; n, no. of samples.

Eight specimens had wild-type *rpoB*, and 2 had mutations not associated with rifampin resistance.

Discussion

We assessed LiPA on the largest reported sample of 1,997 clinical specimens in a nontrial, routine context that would be meaningful to clinicians, especially those submitting samples other than AFB-positive respiratory specimens. The overall unadjusted concordance, sensitivity, specificity, PPV, and NPV were 86.7%, 85.2%, 88.2%, 86.9%, and 86.7%, respectively, for detecting MTBC in primary samples and 98.9%, 98.7%, 100%, 100%, and 93.3%, respectively, for isolates. Previous studies that tested mainly respiratory samples and isolates reported concordance rates with culture from 78.3% to 100% and were usually controlled studies (*8–16*).

When PCR was compared with culture for detecting MTBC, some false-positive results may, in fact, have been true-positive results. Of 118 samples classified as false positive, 83 were believed to be true positive on the basis of our planned protocol. These consisted of 61 samples from patients with a microbiologic diagnosis of MTBC at our laboratory in the last 18 or subsequent 3 months and an additional 22 samples from patients who were receiving antituberculous treatment or who had received it within the last 3 months. Patients who were receiving treatment currently or within the last 3 months were significantly less likely to have MTBC; of 195 samples from such patients, 70 (35.9%) had MTBC compared to 871 (49.3%) of 1,766 samples from patients with no reported treatment within the last 3 months ($\chi^2 = 12.7$, $p<0.001$). Furthermore, a significantly higher proportion of rifampin-resistant MTBC was isolated from patients receiving treatment (12/70, 17.1%) compared with patients not reported to be receiving treatment (34/866, 3.9%, $\chi^2 = 24.2$, $p<0.001$). In these 83 false-positive samples believed to represent true positive results, PCR detected nucleic acid from nonviable organisms (due to treatment) or viable organisms in insufficient numbers for successful culture. If these 83 samples are excluded from overall analysis, specificity improves for all primary specimens, AFB-positive specimens, and AFB-negative specimens from 88.2%, 70.8%, and 95.7%,

Table 5. Results of LiPA in detecting MTBC in clinical specimens in which MTBC was correctly identified and cultured, stratified by history of antituberculous treatment*

Treatment history/ AFB smear result	No. positive/no. tested (%)				
	Concordance	Sensitivity	Specificity	PPV	NPV
Current or within 3 mo	86/182 (47.3)	48/67 (71.6)	38/115 (33.0)	48/125 (38.4)	38/57 (66.7)
Positive	61/132 (46.2)	46/55 (83.6)	15/77 (19.5)	46/108 (42.6)	15/24 (62.5)
Negative/not done	25/50 (50.0)	2/12 (16.7)	23/38 (60.5)	2/17 (11.8)	23/33 (69.7)
Adjusted values†	86/139 (61.9)	48/67 (71.6)	38/72 (52.8)	48/82 (58.5)	38/57 (66.7)
Positive	61/99 (61.6)	46/55 (83.6)	15/44 (34.1)	46/75 (61.3)	15/24 (62.5)
Negative/not done	25/40 (62.5)	2/12 (16.7)	23/28 (82.1)	2/7 (28.6)	23/33 (69.7)
>3 mo ago	85/106 (80.2)	42/53 (79.2)	43/53 (81.1)	42/52 (80.8)	43/54 (79.6)
Positive	54/65 (83.1)	40/45 (88.9)	14/20 (70.0)	40/46 (87.0)	14/19 (73.7)
Negative/not done	31/41 (75.6)	2/8 (25.0)	29/33 (87.9)	2/6 (33.3)	29/35 (82.9)
Adjusted values†	85/102 (83.3)	42/53 (79.2)	43/49 (87.8)	42/48 (87.5)	43/54 (79.6)
Positive	54/63 (85.7)	40/45 (88.9)	14/18 (90.9)	40/44 (90.9)	14/19 (73.7)
Negative/not done	31/39 (79.5)	2/8 (25.0)	29/31 (93.5)	2/4 (50.0)	29/35 (82.5)
No stated treatment	1,497/1,634 (91.6)	692/798 (86.7)	805/836 (96.3)	692/723 (95.7)	805/911 (88.4)
Positive	845/902 (93.7)	661/698 (94.7)	184/204 (90.2)	661/681 (97.1)	184/221 (83.3)
Negative/not done	652/732 (89.1)	31/100 (31.0)	621/632 (98.3)	21/42 (73.8)	621/690 (90.0)
Adjusted values†	1,497/1,634 (91.6)	692/798 (86.7)	805/836 (96.3)	692/723 (95.7)	805/911 (88.4)
Positive	845/892 (94.7)	661/698 (94.7)	184/194 (94.8)	661/671 (98.5)	184/221 (83.3)
Negative/not done	652/728 (89.6)	31/100 (31.0)	621/628 (98.9)	31/38 (81.6)	621/690 (90.0)

*LiPA, line probe assay; MTBC, *Mycobacterium tuberculosis* complex; AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value.
†Excludes samples from patients with a microbiologic diagnosis of MTBC made at the Mycobacterium Reference Unit in the last 18 or subsequent 3 months.

Table 6. Results of LiPA in detecting rifampin resistance in clinical specimens in which MTBC was correctly identified and cultured, stratified by history of antituberculous treatment*

	No. positive/no. tested (%)				
Treatment history	Concordance	Sensitivity	Specificity	PPV	NPV
Current or within 3 mo	46/48 (95.8)	8/10 (80.0)	38/38 (100)	8/8 (100)	38/40 (95.0)
>3 mo ago	41/42 (97.6)	7/7 (100)	34/35 (97.1)	7/8 (87.5)	34/34 (100)
None stated	689/691 (99.7)	23/23 (100)	666/668 (99.7)	23/25 (92.0)	666/666 (100)

*LiPA, line probe assay; MTBC, *Mycobacterium tuberculosis* complex; AFB, acid-fast bacilli; PPV, positive predictive value; NPV, negative predictive value.

respectively, to adjusted values of 96.2%, 91.8% and 97.7% (Table 1). PPV improves from 86.9%, 89.5% and 54.7%, respectively, to 95.7%, 97.5% and 70.0%. Other false-positive samples could probably be excluded; we only chose to exclude those with microbiologic diagnoses of MTBC at our laboratory because we had no data on microbiologic, histologic, or clinical diagnoses made by the other hospitals that submitted these samples. Furthermore, since relevant data were often not provided, many more patients likely were receiving antituberculous therapy that we were unaware of because treatment failure is a common reason for specimens being submitted for testing.

PCR-equivocal results were excluded from the primary analysis. However, a PCR-equivocal result may represent a lack of sensitivity. If PCR-equivocal results are considered PCR negative, the adjusted values for detecting MTBC in primary specimens were only marginally altered to 90.6%, 84.0%, 96.2%, 95.7%, and 85.7%, respectively, for concordance, sensitivity, specificity, PPV, and NPV.

A recent review of LiPA results reported that although little data on clinical specimens were available, sensitivity appeared lower than that of isolates (*10*). Our study confirmed this finding, with sensitivities of 85.2% for all clinical specimens and 98.7% for isolates. As with other PCR-based tests (*21–23*), sensitivities of LiPA for AFB-negative (29.4%) and nonrespiratory samples (61.1%) were low. Sensitivity was also reduced to 71.6% in patients receiving treatment at the time or within 3 months of the time the sample was obtained. Marttila et al. tested 75 clinical specimens with LiPA, including 66 from nonrespiratory sites, and reported a sensitivity of 58.8% compared with final clinical and pathologic diagnoses, whereas cultures showed a sensitivity of 35.3% (*15*). Several factors may explain the lower sensitivity of PCR-based methods in these samples. The mycobacterial load is lower, as demonstrated by the significantly shorter time taken to culture MTBC for AFB-positive samples than for AFB-negative samples (18.5 days vs. 29.5 days, $z = 8.0$, $p<0.001$), and respiratory samples than nonrespiratory samples (18.7 days vs. 25.0 days, $z = 5.6$, $p<0.001$). However, more respiratory samples were AFB positive (94.3% vs. 55.0%). Irregular clumping may take place within paucibacillary specimens, and small, suboptimal sample volumes often lead to sampling errors. Nonrespiratory specimens, especially pleural fluid, bone marrow, pus, and tissue biopsy specimens, may contain inhibitors of amplification (*22*,*23*). Inhibition rates in this study were 1.0% overall, with above-average rates in blood and feces (both 2/3, [66.7%]), pleural fluid (2/110 [1.8%]), bone marrow (1/23 [4.3%]), and pus/tissue (8/400 [2.0%]).

The nonrespiratory specimen types with the highest sensitivity rates were vertebral aspiratesbiopsy specimens (n = 30, sensitivity 83.3%), gastric aspirates (n = 18, sensitivity 80.0%), and lymph node aspirates/biopsy specimens (n = 144, sensitivity 72.5%). For pleural fluid, one of the most commonly submitted samples (n = 107), LiPA had one of the lowest sensitivity rates (21.7%) for detecting MTBC. The difficulties in detecting MTBC in pleural fluid are well recognized, with previous reported sensitivities of 20% (*24*) and 50% (*23*) with the Gen-Probe amplified *M. tuberculosis* direct test.

For detecting rifampin resistance in PCR-positive specimens yielding MTBC on culture, LiPA had concordance, sensitivity, specificity, PPV, and NPV values of 99.1%, 95.0%, 99.6%, 92.7%, and 99.7%, respectively. These results are consistent with previous studies that reported concordance rates of 90.2% to 100% (*8–18*). In this study,

Table 7. Sequence analysis of 10 discrepant rifampin-susceptibility results*

	Rifampin susceptibility		
Sample no.	LiPA result	Phenotypic result	Conclusion after sequencing
1–3	Sensitive	Resistant	From the same patient; wild-type *rpoB* test region
4	Sensitive	Resistant	*Mycobacterium bovis*; wild-type *rpoB* test region
5–7	Resistant (ΔS4)	Sensitive	From the same patient; synonymous substitution (R528R) not associated with rifampin resistance
8	Resistant (ΔS1)	Sensitive	2 genotypes present: wild-type (predominant) and mutant (L511P)
9	Resistant (R5)	Sensitive	S531L mutation; wild-type *rpoB* on retesting, thus likely laboratory error
10	Resistant (ΔS2)	Sensitive	D516A mutation; no high-level resistance when seen alone

*LiPA, line probe assay.

of the 69 rifampin-resistant MTBC strains cultured, 5 were PCR negative for MTBC. Of the remaining 64 that were PCR positive, 59 (93.7%) had detectable *rpoB* mutations and were reported as resistant. At least 90% of rifampin-resistant strains have mutations within the target *rpoB* region, although this proportion may vary in different populations (*7*).

Detection of rifampin resistance by LiPA may be used as an early predictor of MDRTB before phenotypic susceptibilities are available, but this clearly depends on the prevalence of rifampin monoresistance in the study population. The diagnosis of rifampin monoresistance is also critical because this automatically invalidates the use of short-course chemotherapy (*25*). Of the 59 correctly identified rifampin-resistant MTBC isolates, 11 were rifampin monoresistant. The overall prevalence was 1.0% in this study, which was higher than the 0.3% reported in a national UK survey (*6*). This result reflects a common underlying reason for specimen referral for Fastrack analysis, i.e., failure of response to treatment.

For primary samples in which LiPA detected MTBC, diagnosis of tuberculosis was made an average of 15.2 days earlier than with automated liquid culture (14.8 days for AFB-positive specimens and 22.1 days for AFB-negative specimens). More days were saved with nonrespiratory samples (18.3 days) than with respiratory samples (14.7 days), although these samples had the lowest probability of detection. LiPA accurately determined rifampin susceptibility earlier than solid culture-based techniques by a mean of 30.7 days for all primary specimens. This compares favorably with a study that found that LiPA saved a median of 24 days compared with susceptibility testing with the BACTEC liquid culture system (Becton Dickinson, Sparks, MD, USA) and 54 days with solid media (*11*).

In summary, LiPA may be used with clinical samples for diagnosis of MTBC and rifampin resistance, saving, when positive results are obtained, an average of 15.2 days and 30.7 days, respectively, compared with conventional techniques. However, some limitations of LiPA are evident. As with other PCR-based assays, sensitivity is reduced in AFB-negative and nonrespiratory samples, such as paucibacillary forms of the disease, in which rapid diagnosis would be most helpful. Although the assay is a potential diagnostic route for patients receiving therapy, sensitivity is also reduced in these circumstances. The lower sensitivity rates for certain samples and the possibility of a PCR-equivocal or PCR-inhibited result also mean that conventional culture and sensitivity testing should still be used at the same time. Alternatives to LiPA may be useful, e.g., we used an IS*6110*-based PCR for diagnosis of tuberculous meningitis. Similarly, rifampin-resistance mutations can be detected by DNA sequencing (we now sequence all PCR products identified as MTBC with any form of rifampin probe mutations) or with noncommercial macroarrays (*26*,*27*). Thus, molecular results, as with any laboratory test, should be reviewed in the context of all clinical, microbiologic, and histologic results.

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Multidrug-resistant Tuberculosis in Military Recruits

Grace Freier,* Allen Wright,* Gregory Nelson,* Eric Brenner,† Sundari Mase,‡ Sybil Tasker,§ Karen L. Matthews,¶ and Bruce K. Bohnker¶

We conducted a tuberculosis contact investigation for a female military recruit with an unreported history of multidrug-resistant tuberculosis (MDRTB) and subsequent recurrence. Pertinent issues included identification of likely contacts from separate training phases, uncertainty on latent MDRTB infection treatment regimens and side effects, and subsequent dispersal of the contacts after exposure.

In 2004, a 19-year-old female recruit came to the Naval Hospital in Beaufort, South Carolina, with a history of congestion and rhinorrhea for 4 days. Radiographic examination showed right upper and lower lobe infiltrates. Her initial recruit screening tuberculin skin test (TST) result had been reactive. Consultation with her physician in California indicated similar radiographic findings 2 years earlier; her condition had been diagnosed as smear- and culture-negative tuberculosis (TB). She received oral treatment of 300 mg isoniazid daily, 600 mg rifampin daily, and 1,500 mg pyrazinamide daily for 2 months. After a negative sputum culture, isoniazid and rifampin were continued for 9 months (*1*,*2*). Based on unchanged radiographic findings and 9 months of treatment, her disease was considered to be nonactive and she returned to training. Subsequently, she failed to complete training and was separated from the military.

The Study

Approximately 3 months after her initial treatment, the index patient was hospitalized in California for TB resistant to isoniazid and rifampin, which met the definition of multidrug-resistant tuberculosis (MDRTB). Initial isolate susceptibility in California showed resistance to isoniazid, rifampin, ethambutol, and streptomycin. Additional isolate susceptibility tests in Denver showed sensitivity to ethionamide, cycloserine, p-amino salicylic acid, clofazimine, levofloxacin, and pyrazinamide, but resistance to isoniazid, rifampin, streptomycin, amikacin/kanamycin, amoxicillin/clavulanate, and rifabutin.

After notification of the recruit's hospitalization in California, Navy personnel began a TB contact investigation (*1*). Recruit populations are highly transient, as persons are frequently added or removed for various medical, dental, legal, or physical performance reasons. Some persons had multiple exposures to the index patient while in the training platoon and subsequently in various processing units. Thus, the contact investigation identified numerous persons who may have had contact with the index patient; these were categorized as "close" or "casual" contacts. Close contacts included persons who shared living quarters with the index patient; casual contacts included persons who had less definable contact with the index patient.

The investigation identified 13 close contact and 8 casual contact new reactors, defined as ≥5 mm TST indurations in persons who had negative tests previously (*2*). These persons were considered likely to have been infected with the MDRTB strain, though none demonstrated active disease. Table 1 shows that the close contact group had a TST reactor proportion of 9.09%. Table 2 shows a 3.1% TST reactor rate for the casual contact group (risk ratio [RR] 2.86, 95% confidence interval [CI] 1.22–6.74, p = 0.011). The index patient was assigned to the recruit-training platoon for 3 weeks, a rehabilitation squad for 9 days, and the separation platoon for 4 days. The TST reactor proportion for persons with >3 weeks of exposure in the recruit-training platoon was substantially lower than shorter duration of exposure in the rehabilitation and separation units (RR 0.19, 95% CI 0.05–0.66, p = 0.0032). A possible explanation for this apparent paradox would be increasing infectiousness during this later period, which is supported by progressive clinical symptoms seen in the index patient.

The optimal treatment protocol for new TST reactors from likely MDRTB sources is undefined, which leads to extensive consultation with TB experts to determine treatment timing and medications (*3*–*10*). The imminent transfer of reactors to new duty stations and the upcoming holiday leave period complicated the recommendations. Timing options included the following: 1) start medication immediately, retain reactors on base 7–10 days to verify medication tolerance, and allow self-medication during the transfer to their next duty station; 2) start medication immediately, allow self-medication during holiday leave, and continue therapy at their next duty station; or 3) delay treatment until reactors complete 2–3 weeks of holiday leave and initiate treatment at their next duty station. Ultimately, the graduating recruits were allowed holiday leave and began therapy at their next duty station.

Because the index patient's isolate was resistant to isoniazid and rifampin, several medication options were

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Table 1. Close contact tuberculin skin test (TST) reactor rates by exposure location

	Total	Old reactors	TST	New reactor	Reactor rate (%)
Recruit training platoon only	67	1	65	4	6.15
Recruit processing units only	53	1	48	5	10.04
Multiple exposures	38		30	4	13.33
All close contacts	158	2	143	13	9.09

considered. The literature was reviewed and options assessed for medications, adverse effects monitoring (clinical vs. biochemical), duration (4, 6, 9, 12, or 24 months), and self-administered versus directly observed therapy. Three options emerged: 1) no medication with close clinical and radiologic monitoring for 2–3 years; 2) monotherapy with a fluoroquinolone; or 3) two-drug regimen consisting of pyrazinamide and a fluoroquinolone. This third option, initially strongly considered from prior recommendations (*4*), was not chosen because published case series suggested poor tolerance and unacceptable hepatotoxicity (*8*,*9*). By consensus, US Navy and Centers for Disease Control and Prevention (CDC) infectious disease specialists recommended a fluoroquinolone for at least 12 months. In vitro studies suggest that gatifloxacin and moxifloxacin have greater activity against *Mycobacterium tuberculosis* than older fluoroquinolones, though treatment efficacy for latent TB infection has not been documented in the literature (*11*,*12*). Ultimately, gatifloxacin was selected based on availability on the Department of Defense formulary. Therefore, the recruit reactors at high risk for latent TB infection from the MDRTB isolate were counseled, and 400 mg gatifloxacin was administered orally daily. Although the Food and Drug Administration had not approved gatifloxacin to treat TB, this protocol represented the most appropriate therapy, based on the limited data available.

Upon arrival for training, recruits receive a single-step TST and have historically demonstrated a baseline TST reactor proportion of 0.35% (*13*). However, several of the reactors in the casual exposure category were not recruits and had vague and limited exposure histories. For example, 1 reactor drove a bus that the index patient may have ridden. Persons in these positions do not routinely undergo TST screening and would be in populations with unknown TST conversion rates. Using the "concentric ring approach," further investigation on base was deferred since the conversion proportion of personnel with positive TST results could not be separated from the background level in the local population (*2*). Military personnel would continue to receive TST surveillance consistent with the most current Navy medicine policy (*1*).

Only 6 of the 13 reactors in the higher-risk groups remained on active duty, and their transfer required explicit coordination to ensure appropriate follow-up. In collaboration with CDC, military preventive medicine personnel communicated with 5 state departments of health to ensure appropriate follow-up for the other 7 TST reactors in the high-risk group. More than 30 state health departments were notified of other casual contacts that were dropped from training.

Conclusions

This contact investigation illustrates the complexities associated with the public health management of MDRTB exposures in military recruit training settings. It demonstrates the importance of close coordination of efforts among military medical personnel, expert tuberculosis consultants, CDC, and state health departments in such cases. It shows some of the uncertainties in the clinical management of reactors associated with exposure to MDRTB sources, exacerbated in this case by military related factors. It highlights the complexities associated with public health management of MDRTB exposure and demonstrates the necessity of response preparedness, close consultation, communication, and coordination of efforts.

Table 2. Tuberculin skin test (TST) reactor rate by exposure duration

	Contact duration	Total	Old reactors	TSTs placed	New reactor	Reactor rate (%)	Group reactor rate (%)
Casual contacts*	Likely none	25	0	19	1	5.26	3.2
	Possible	256	13	233	7	3.00	
Close contacts	Unknown	34	1	33	1	3.03	9.09
	>3 weeks† Sep 13–Oct 12	70	1	70	3	4.29	
	1–3 weeks Oct 12–21	42	0	31	6	19.35	
	<1 week Oct 21–26	12	0	9	3	33.33	
Total		439	15	395	21	5.32	

*The close contacts were more likely to convert than the incidental contacts. Risk ratio (RR) 2.86, 95% confidence interval (CI) 1.22–6.74, p = 0.011.
†The close contacts with >3 weeks of exposure were less likely to convert than those with <3 weeks of exposure. RR 0.19, 95% CI 0.05–0.66, p = 0.0032.

This outbreak preceded recently published guidance on TB investigations and treatment, although it was generally handled consistent with that guidance (*14*,*15*).

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Mycobacterium bovis Isolates with *M. tuberculosis* Specific Characteristics

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Our study is the first report of exceptional *Mycobacterium bovis* strains that have some characteristics of *M. tuberculosis*. The strains were isolated from 8 patients living in Kazakhstan. While molecular markers were typical for *M. bovis*, growth characteristics and biochemical test results were intermediate between *M. bovis* and *M. tuberculosis*.

Mycobacterium bovis causes tuberculosis (TB) mainly in cattle but has a broad host range and causes disease similar to that caused by *M. tuberculosis* in humans (*1*). It belongs to the *M. tuberculosis* complex (MTBC) that comprises the closely related human pathogens *M. tuberculosis* and *M. africanum* (*2*). Identification of *M. bovis* traditionally has been based on clear-cut differences in phenotypic characteristics and biochemical properties when compared to the other members of the MTBC (*1,2*). *M. bovis* shows a dysgonic colony shape on Lowenstein-Jensen medium, is negative for niacin accumulation and nitrate reduction, is susceptible to thiophene-2-carboxylic acid hydrazide (TCH), and shows microaerophilic growth on Lebek medium (*1–3*). A further criterion used for differentiation is the intrinsic resistance to pyrazinamide, which is found in most *M. bovis* isolates (*1–3*). In contrast, *M. tuberculosis* shows eugonic growth, is positive for niacin accumulation and nitrate reduction, is resistant to TCH, shows aerophilic growth on Lebek medium, and is usually not monoresistant to pyrazinamide (*2,3*).

More recently, several molecular methods have been developed that provide clear criteria for the identification of *M. bovis*. These comprise a variety of polymerase chain reaction (PCR) methods, e.g., based on DNA sequence variations in the direct repeat region of MTBC complex strains (spoligotyping [*4*]) or on single nucleotide polymorphisms (SNPs) in either the *oxyR* gene (*5*) or the *gyrB* gene (*6*). Furthermore, MTBC isolates can be differentiated by large sequence polymorphisms or regions of difference (RD), and according to their distribution in the genome, a new phylogenetic scenario for the different species of the MTBC has been suggested (*7–9*). The presence or absence of particular deletions has been proposed as being discriminative, e.g., lack of TdB1 for *M. tuberculosis* or lack of RD12 for *M. bovis*.

In routine diagnostics, the combination of phenotypic characteristics and biochemical features is sufficient to differentiate clinical *M. bovis* isolates, and in general, the results obtained are unambiguous. However, here we describe the characteristics of 8 strains of the MTBC that showed an unusual combination of phenotypic and biochemical attributes of both *M. bovis* and *M. tuberculosis*. Molecular analyses confirmed the strains as *M. bovis*, which in part have phenotypic and biochemical properties of *M. tuberculosis*.

The Study

During a previous investigation of 179 drug-resistant isolates from Kazakhstan (*10*), we determined the presence of 8 strains showing monoresistance to pyrazinamide. Kazakhstan is the largest of the central Asian republics and is divided regionally into 14 oblasts. The investigation was performed as part of a nationwide drug resistance survey conducted by the national TB program of Kazakhstan with assistance from the World Health Organization/ International Union against Tuberculosis and Lung Disease Global Project in 2001. The subset of this survey investigated here (n = 158) represents 100% of strains resistant to isoniazid, rifampin, ethambutol, or streptomycin isolated in 9 of the 14 Kazakhstan oblasts during the study period; 21 samples had fungal contamination or showed no growth (*10*). All strains were isolated from sputum samples.

To further clarify if these strains were monoresistant *M. tuberculosis* or *M. bovis* isolates intrinsically resistant to pyrazinamide, we performed several routine diagnostic tests traditionally used for species differentiation (*6*). All strains showed eugonic growth characteristics on Lowenstein-Jensen slants and on bromcresol purple medium (Figure), which in general is typical for *M. tuberculosis*. However, on bromcresol purple medium, classic *M. tuberculosis* isolates induce a pH-dependent change of color from blue to yellow, which was not observed in these cases (Figure). Furthermore, all 8 isolates were positive for niacin accumulation, negative for nitrate reduction, susceptible to TCH, and showed aerophilic growth on Lebek medium. Considering all results, the 8 strains showed a combination of test results that did not allow a clear differentiation as *M. bovis* or as *M. tuberculosis* (Table 1). Such a combination of test results would apply best to *M. africanum*, a species

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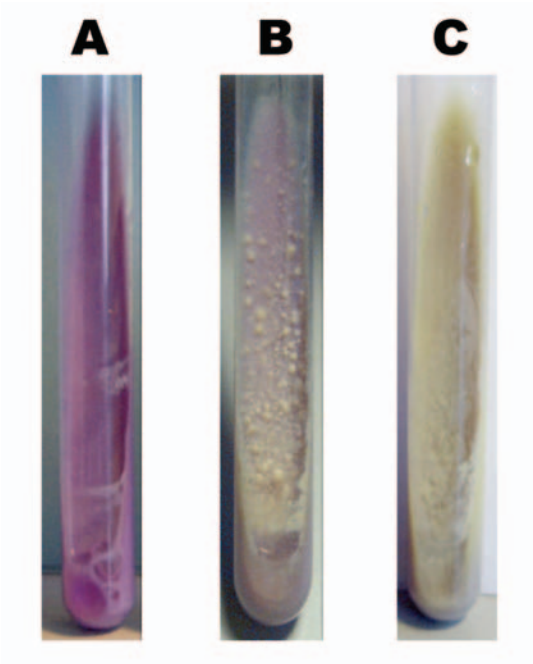

Figure. Growth morphology on bromcresol purple medium of *Mycobacterium bovis* (A), *M. tuberculosis* (C), and 1 of the strains analyzed (B).

from which more variable test results have been reported (*3*). However, this species was probably was not isolated because *M. africanum* strains are usually not monoresistant to pyrazinamide (3).

Therefore, we investigated all strains with several molecular techniques previously used for differentiation within the MTBC (Table 2). They all had identical spoligotype patterns (hexcode 6B-57-5F-7F-FF-60, performed according to the methods of Kamerbeek et al. [*4*]), that lacked spacers 39–43 and identical IS*6110* DNA fingerprint patterns with 2 IS*6110* copies (data not shown, performed according to the methods of van Embden et al. [*11*]). All isolates carried the *M. bovis*–specific polymorphism in the *oxyR* gene (*5*), and none of them had the *M. bovis* BCG– specific deletion in the RD1 region (*12*). PCR analysis of other RDs (RD3, RD4, RD5, RD9, RD10, RD12, TbD1, and IS*1541*) showed results typical for *M. bovis* when compared with the RD signatures of the American Type Culture Collection strains of *M. tuberculosis*, *M. africanum*, and *M. bovis* (Table 2) and with previously published data (*7*,*8*). The intrinsic resistance to pyrazinamide was confirmed by DNA sequence analysis as all strains carried the *M. bovis*–specific single point mutation at nucleotide position 169 of the *pncA* gene.

Seven of the 8 strains were isolated from 30- to 55-year-old men, and 1 strain was from a 72-year-old woman. All but 1 patient had a history of previous antituberculosis treatment, but none of the strains showed any further resistance (data not shown). The patients originated from the oblast of Kostanajskaya in north Kazakhstan. Among all patients, no direct epidemiologic links could be established. However, 3 of the patients lived in the city of Kostanaj, while 5 came from rural areas. Before 1950, the Kazakh Steppe was a broad, continuous belt of grassland that stretched from the Ural River to the Altai foothills, covering large parts of Kostanajskaya; after the 1950s, the region was used extensively for agriculture. Information on contact with animals is not available, since cattle herds are only kept privately.

Conclusions

We describe 8 strains of *M. bovis* with exceptional phenotypic characteristics that are intermediate between *M. tuberculosis* and *M. bovis*. This fact initially complicated a clear species differentiation; however, the battery of molecular tests performed clearly confirmed all strains as *M. bovis*. These tests included the presence of characteristic single nucleotide polymorphisms as well as an RD profile that is typical for the *M. bovis* lineage of the MTBC (*6*,*7*). To our knowledge, this is the first report describing *M. bovis* isolates with phenotypic characteristics and biochemical properties of *M. tuberculosis*. In our previous investigation of 176 *M. bovis* strains from Germany, all strains had phenotypic characteristics typical of *M. bovis*, and no strains similar to the isolates from Kazakhstan could be identified (*13*). The same result applies for the

Table 1. Phenotypic characteristics of type strains *Mycobacterium tuberculosis* H37 (ATCC 27294), *M. bovis* (ATCC19210), *M. africanum* (ATCC25420), and the strains analyzed*

Strains	Colony morphology†	Test result					Growth on Lebek medium
		TCH‡	PZA	Niacin accumulation	Nitrate reduction	Change of color of bromcresol medium	
Kazakhstan (n = 8)§	Eugonic	S	R	+	–	–	Aerophilic
ATCC *M. bovis*	Dysgonic	S	R	–	–	–	Microaerophilic
ATCC H37	Eugonic	R	S	+	+	+	Aerophilic
ATCC *M. africanum*	Dysgonic	S	S	+	+	–	Microaerophilic

*TCH, thiophene-2-carboxylic acid hydrazide; PZA, pyrazinamide; +, positive test result; –, negative test result; S, susceptible; R, resistant.
†Growth characteristics on Lowenstein-Jensen and bromcresol purple slants.
‡Growth in presence of TCH.
§All 8 strains showed identical test results.

Table 2. Genetic characteristics of type strains *Mycobacterium tuberculosis* H37 (ATCC 27294), *M. bovis* (ATCC19210), *M. africanum* (ATCC25420), and the strains analyzed*

Strains	Test result										
	TbD1	RD1	RD3	RD4	RD5	RD9	RD10	RD12	*IS1561*	*oxyR*†	*gyrB*‡
Kazakhstan (n = 8)§	1	1	1	0	0	0	0	0	1	1	*M. bovis*
M. bovis ATCC	1	1	1	0	0	0	0	0	1	1	*M. bovis*
M. tuberculosis H37	0	1	1	1	1	1	1	1	1	0	*M. tuberculosis*
M. africanum ATCC	1	1	0	1	1	0	0	1	1	0	*M. africanum*

*RD, region of difference; 0, region deleted; 1, region present.
†Presence of *oxyR* mutation G to A at position 285; 1, polymorphism present; 0, polymorphism not present.
‡Classification according to *gyrb*-polymerase chain reaction restriction fragment length polymorphism analysis (Niemann et al. [*3*]).
§All 8 strains showed identical test results.

spoligotype patterns, as none of the strains in our database had an identical spoligotype pattern (data not shown). A further comparison with the international *M. bovis* spoligotype database (available from http://www.mbovis.org/spoligodatabase) identified 1 strain isolated in Argentina with an identical spoligotype pattern; however, no further information about his strain is available.

Whether the 8 strains analyzed represent strains of an ancestral phylogenetic lineage of *M. bovis* that might have been conserved because of the geographic isolation of that region of Kazakhstan or whether they gained their special characteristics by new mutations is a question that cannot be answered by the data obtained in this study. All strains have been isolated from humans. We cannot say if we have found an exceptional outbreak of a particular *M. bovis* strain or if the patients were infected directly by wildlife, livestock, or food, and the disease developed by chance during the study. However, an overall percentage of ≈5% of all resistant strains investigated in this study indicates that these isolates may be important in Kazakhstan. This also poses the question of whether these strains might become more virulent in humans if they acquired phenotypic/biochemical characteristics usually observed exclusively in *M. tuberculosis*. However, to address this question more precisely, longitudinal studies on the population structure of MTBC isolates in Kazakhstan obtained from humans and animals, in combination with experiments in virulence model systems, will be necessary. In any case, these strains represent ideal model organisms for analyzing the nature of the biologic differences observed between *M. bovis* and *M. tuberculosis*. To ensure a clear differentiation from other *M. bovis* strains, we suggest the name *M. bovis* subtype "Almaty" for this genotype. Almaty is the former capital and largest city of Kazakhstan.

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Tuberculosis-HIV Co-infection in Kiev City, Ukraine

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In 2004, we tested all patients with newly diagnosed tuberculosis (TB) for HIV in Kiev City. The results were compared to information from medical records of 2002, when co-infection prevalence was 6.3%. Of 968 TB patients, 98 (10.1%) were HIV infected. TB-HIV co-infection is increasing, especially in injecting drug users.

In Ukraine, the prevalence of HIV infection has been increasing since the mid-1990s (*1*,*2*). By January 2005, a total of 74,856 cases of HIV infection had officially been registered (http://www.aidsalliance.kiev.ua/). The real number of HIV infected persons may be much higher, an estimated 330,000–410,000 in 2001 (>1% of adult population) (*3*).

An increase in HIV prevalence is usually closely followed by an increase in tuberculosis (TB) (*4*). In Kiev City, the number of TB patients registered for treatment doubled from 629 in 1992 to 1,274 in 2004. This increase is mainly explained by economic and social changes after independence (August 1991), but the progressing HIV epidemic may also play a role in the increase in the number of TB patients.

In a previous study in Kiev City, we estimated the prevalence of HIV infection in patients with newly diagnosed TB at 6.3% in 2002 (*5*). In this study, we assess the prevalence of HIV infection ≈2 years later and compare the results of the 2 studies. We also determine risk factors for TB-HIV co-infection.

The Study

From March 2004 to February 2005, all patients with newly diagnosed TB, who were ≥18 years of age and living in Kiev City, and who had begun anti-TB chemotherapy in the Kiev City TB Services were eligible for inclusion. Patients were informed about the study, counseled, and asked to participate. Basic information was collected about all TB patients from medical records and by interview. Reasons for not providing a blood sample for HIV testing were also recorded.

In Ukraine, TB diagnosis is made by smear and culture examination. All persons with suspected TB are evaluated by a committee of experts. TB is classified as pulmonary TB bacteriologically confirmed (smear or culture positive), pulmonary TB bacteriologically not confirmed (smear and culture negative or not done), and extrapulmonary TB.

Blood samples were tested for HIV by using Genscreen Plus HIV Ag-Ab (Bio-Rad Laboratories, Steenvoorde, France). Confirmation of the test result was done by Abbott IMx system HIV-1/-2 3rd Generation Plus (M/S Abbott GmbH, Wiesbaden, Germany). We tried to retest cases with an indeterminate HIV test result. TB patients with a positive test result were referred to the Kiev Anti-AIDS Centre.

We used SPSS 12.0 (SPSS Inc., Chicago, IL, USA) for data analysis with *t* tests and χ^2 tests. Differences at the $\alpha = 5\%$ level were regarded as significant. We examined predictive factors for HIV infection by logistic regression. The results were compared to those of a study using medical record information from patients newly diagnosed with TB in Kiev City in 2002 (*5*).

The study was approved by the medical ethics committee of the Yanovskiy Institute of Phtisiology and Pulmonology, Kiev City, Ukraine. Written informed consent was obtained from all participants.

A total of 1,090 TB patients were included from the 9 TB clinics and hospitals in Kiev City. Of those 1,090 TB patients, 4 (0.4%) could not be counseled because they were too ill or intellectually impaired, 83 (7.6%) did not provide informed consent, and 15 (1.4%) had to be excluded from the study, primarily because medical workers could not obtain a blood sample. The 102 (9.4%) TB patients who did not participate in the study were significantly older than those included: mean ages, respectively, 45.4 and 39.1 years ($p<0.001$). Other characteristics were not significantly different.

Of the 988 TB patients tested for HIV infection, 33 (3.3%) had an initial indeterminate test result. Sixteen of those were retested, 3 refused retesting, and 14 were not approached. Of those retested, 12 tested negative, 1 tested positive, and 3 again had an indeterminate test result. Thus, 968 TB patients with a definite HIV test result could be included in the analysis. Of those, 98 (10.1%) were HIV infected, 64 (65.3%) were identified in our study as HIV infected, and 34 (34.7%) had received a diagnosis of HIV infection from the Kiev Anti-AIDS Centre laboratory before being referred to the TB services with suspected TB.

Reported injecting drug use was the strongest independent predictor for HIV infection (Table 1). Those reporting injecting drug use were 31.4 times more likely to

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Table 1. Risk factors for a positive HIV test in patients with newly diagnosed TB in Kiev City, Ukraine*

Variable	No. (% HIV infected)	Univariate, OR (95% CI)	Multivariate, OR (95% CI)
Sex			
Male	712 (11.0)	1	
Female	256 (7.8)	0.69 (0.41–1.15)	
Age, y			
18–29	318 (14.8)	1	1
30–39	224 (17.4)	1.22 (0.76–1.93)	1.69 (0.94–3.04)
40–49	195 (4.6)	0.28 (0.13–0.58)	0.56 (0.24–1.30)
≥50	231 (1.3)	0.08 (0.02–0.25)	0.18 (0.05–0.62)
Classification			
PTB+	541 (10.5)	1	
PTB-	379 (8.2)	0.76 (0.48–1.20)	
EPTB	48 (20.8)	2.24 (1.06–4.72)	
STD in last 5 y			
No	880 (8.6)	1	1
Yes	27 (22.2)	3.02 (1.18–7.72)	4.41 (1.57–12.38)
Unknown	61 (26.2)	3.76 (2.03–7.00)	1.99 (0.84–4.71)
Homeless			
Yes	56 (12.5)	1.29 (0.57–2.93)	
No	912 (10.0)	1	
Injecting drug use			
Yes	84 (66.7)	40.10 (23.15–69.45)	31.42 (17.35–56.87)
No	884 (4.8)	1	1
Abuse of alcohol			
Yes	105 (10.5)	1.04 (0.54–2.03)	
No	863 (10.1)	1	
Incarcerated >1994			
Yes	117 (15.1)	1.75 (1.01–3.05)	
No	851 (9.4)	1	

*n = 968; TB, tuberculosis; OR, odds ratio; CI, confidence interval; STD, sexually transmitted disease; PTB+, pulmonary TB bacteriologically confirmed; PTB–, pulmonary TB bacteriologically not confirmed; EPTB, extrapulmonary TB.

be HIV infected than those not reporting injecting drug use (95% confidence interval [CI] 17.4–56.9). Also, those who had reported a sexually transmitted disease in the past 5 years were more often HIV infected (odds ratio [OR] 4.4, 95% CI 1.6–12.4).

The prevalence of HIV infection among TB patients significantly increased from 6.3% in 2002 to 10.1% from March 2004 through February 2005 (p = 0.011) (Table 2). The prevalence of HIV-infected TB patients who reported injecting drug use increased from 1.8% of all tested patients with newly diagnosed TB in 2002 to 5.8% in March 2004 through February 2005. Thus, the main increase in TB-HIV co-infection was attributable to an increase in TB-HIV co-infected patients who reported injecting drug use. A larger proportion of persons with a positive HIV test result reported injecting drug use in 2004 (57.1%) than in 2002 (27.8%) (p = 0.003).

HIV co-infection prevalence may be slightly overestimated in the 2002 study (*5*). In the study conducted between March 2004 and February 2005, TB patients included in the study were more frequently <50 years of age (p<0.001). TB patients <50 years of age were more frequently HIV infected. Both studies may therefore overestimate the prevalence of TB-HIV co-infection.

Conclusions

HIV infection increased in patients with newly diagnosed TB in Kiev City between 2002 and 2004. This finding is in agreement with the increase in the number of registered cases of HIV infection in Ukraine since 1995 (*6*).

The main risk factor for being co-infected with HIV was reported injecting drug use. In 2002, 62.5% of the TB patients that reported injecting drug use were HIV infected and in 2004 this number was 66.7%. In Ukraine, the HIV epidemic started in injecting drug users thus that the main risk factor for HIV infection was injecting drug use is not surprising.

We used voluntary confidential HIV testing. Previous studies have found that use of this testing method can result in participation bias because those at higher risk of infection are more likely not to contribute specimens (*7–11*) or selection bias if clinicians encourage testing in those they consider to be more at risk (*5*). Although unlinked anonymous testing would have prevented these problems, the TB physicians participating in the study believed that using this strategy was not feasible. In our study, 7.6% refused to provide informed consent. This finding is comparable to researchers' experiences in other countries (*7,11,12*).

Table 2. Comparison of TB patients tested for HIV in 2002 and 2004

Variable	2002 study, n = 567 (%)	2004 study, n = 968 (%)	p
HIV infected			0.011
Yes	36 (6.3)	98 (10.1)	
No	531 (93.7)	870 (89.9)	
Sex			0.720
Male	412 (72.7)	712 (73.6)	
Female	155 (27.3)	256 (26.4)	
Age, y			0.091
0–29	153 (27.0)	318 (32.9)	
30–39	133 (23.5)	224 (23.1)	
40–49	131 (23.1)	195 (20.1)	
≥50	150 (26.5)	231 (23.9)	
Classification*			0.002
PTB+	360 (63.5)	541 (55.9)	
PTB–	172 (30.3)	379 (39.2)	
EPTB	35 (6.2)	48 (5.0)	
Homeless			0.508
Yes	38 (6.7)	56 (5.8)	
No	529 (93.3)	912 (94.2)	
Injecting drug user			<0.001
Yes	16 (2.8)	84 (8.7)	
No	551 (97.2)	884 (91.3)	
Abuse of alcohol			0.020
Yes	85 (15.0)	105 (10.8)	
No	482 (85.0)	863 (89.2)	
Ever incarcerated			<0.001
Yes	40 (7.1)	132 (13.6)	
No	527 (92.9)	836 (86.4)	

*TB, tuberculosis; PTB+, pulmonary TB bacteriologically confirmed; PTB–, pulmonary TB bacteriologically not confirmed; EPTB, extrapulmonary TB.

In the 2002 study, HIV testing was performed with a locally produced HIV test with unknown specificity and sensitivity. We do not know whether the estimated co-infection prevalence is valid or if we are likely to underestimate the true co-infection prevalence. Even if the locally produced HIV test did not correctly identify 12% of the HIV-positive patients (sensitivity 88%), the prevalence of TB-HIV co-infection was still significantly higher in 2004.

In the 2002 study, the percentage of persons not tested was high (38.0%), compared to a proportion of 10.5% in the 2004 study. If we assume that no HIV infections existed among those not tested, the minimum HIV-infection prevalence in the 2002 study is 3.9% and in the 2004 study 9.1%. No notable differences were identified between those tested and those not tested in variables that were strongly associated with HIV infection. In conclusion, TB-HIV co-infection is increasing in Kiev City, especially in injecting drug users.

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We acknowledge the cooperation of the physicians of the Kiev City Tuberculosis Services for including TB patients in the study and providing data.

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Mycobacterium bovis Isolates from Tuberculous Lesions in Chadian Zebu Carcasses

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This slaughterhouse study in Chad shows higher proportions of *Mycobacterium bovis* isolates among Mbororo than Arabe zebu cattle. Spoligotyping shows a homogenetic population structure for *M. bovis* and lack of spacer 30, as were found in neighboring Cameroon and Nigeria. This finding suggests transborder and ongoing transmission between cattle.

In Chad, prevalences of tuberculin-positive cattle are 0.8% (95% confidence interval [CI] 0.2%–1.4%) in the east (Ouaddaï region) (*1*) and 16.9% (95% CI 10.4%–23.5%) in the west (Chari-Baguirmi and Kanem regions) (*2*). The latter comparative intradermal tuberculin study was conducted with 34 additional transhumant herds; a prevalence of 11.5% (CI 6.9%–18.5%) was found when herds were considered as random effect in the model. More tuberculin reactors were found among Mbororo than Arab zebus (p = 0.02). In the slaughterhouse of Farcha in N'Djaména, 90% of slaughtered cattle are of the Arab zebu breed, 7% Mbororo zebu, and 3% Kouri (*3*). Previous slaughterhouse studies showed that bovine tuberculosis (TB) is an important cause of condemnation (i.e., if a carcass is fully condemned, the whole carcass is destroyed [≈9% of all inspected cattle carcasses]) (*4*). A retrospective study on causes of condemnation after meat inspection showed that most carcasses with tuberculous lesions were detected from July to November and that more Mbororo cattle than other breeds had TB-like lesions (42/60 vs. 132/1,539) (*5*). The diagnosis of suspected bovine TB was based on sighting of typical macroscopic lesions of the organs during meat inspection.

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In Chad, until this study was undertaken, bovine TB was not confirmed by isolation or molecular characterization of the causative agent, *Mycobacterium bovis*. This organism is recognized as a zoonotic pathogen that infects many persons, particularly in the developing world. The highest prevalence of coinfection with bovine TB and HIV/AIDS is also in the developing world (*6*). Our study was aimed at isolating the first *M. bovis* isolates from specimens of Mbororo and Arab cattle in the newly setup mycobacteriology unit of the veterinary laboratory of Fracha, at characterizing the isolates with molecular methods, and at comparing the isolates with those from Cameroon (*7*).

The Study

From July 1 to August 31, 2002, a total of 727 of 10,000 cattle carcasses at the slaughterhouse of Farcha were condemned because of TB-like lesions on meat inspection. The overall prevalence of suspect lesions was 7.3%. A significantly higher (p = 0.04) proportion of lesions was found among Mbororo (8.2%; 212/2,596) than Arab (7%; 515/7,397) cattle (*8*). Lesions were mainly found in the lymph nodes and lungs (Table).

Specimens from 201 affected organs (lymph nodes, lungs, and liver) of 199 randomly selected carcasses were collected for further processing along with the following information: breed, sex, partial or total condemnation of the carcass, date of collection, and nature of specimen (*8*). The geographic origins of the cattle could not be evaluated as they were brought to the slaughterhouse by traders from local livestock markets. In the subsample of 199 animals, entire condemnation of the carcass in comparison to partial condemnation occurred more often among Mbororo than Arab cattle (19/75 vs. 11/124, χ^2, p = 0.002). A higher proportion of Mbororo cattle with bovine TB infection was also observed in Cameroon (*9*); this finding may indicate that Mbororo are more susceptible to *M. bovis* strains in the 2 Central African countries.

The 201 collected specimens were washed 3 times with sterile, distilled water. Tissue samples were cut into 5 or 6 pieces and put in a sterile plastic bag containing 10 mL sterile saline for homogenization. Samples were homogenized in a blender for 1 min; this process was repeated 3 times. Ten milliliters of the suspension was decontaminated with N-acetyl-L-cysteine sodium hydroxide (0.5% NALC–2% NaOH) (*10*), and 0.25mL was injected onto 2 Lowenstein-Jensen slants, l containing glycerol (0.75%) and l containing pyruvate (0.6%). In addition, Middlebrook 7H9 medium containing oleic acid-albumin-dextrose-catalase and PANTA (polymyxin, amphotericin B, nalidixic acid, trimethoprim, azlocillin) were injected

[1]These authors contributed equally to this study.

Table. Specimens collected at the main slaughterhouse of N'Djaména, Chad, and specifications of the condemned carcasses

Organ/tissue	n	Condemnation		Breed		Sex	
		Entire	Partial	Arab	Mbororo	Male	Female
Lymph nodes	116	17	99	67	49	8	108
Lungs	75	13	62	51	24	1	74
Lungs and lymph nodes	2	0	2	2	0	0	2
Liver	5	0	5	4	1	0	5
Miliary tuberculosis	1	0	1	0	1	0	1
Total	199	30	169	124	75	9	190

with 0.5mL of the decontaminated suspension. Injected media were incubated at 37°C (without CO_2) for 8 weeks. Growth of mycobacteria was confirmed by smear (stained by the Ziehl-Neelsen method) and acid-fast–positive colonies were subcultured. Three biochemical tests (*11*) were used to distinguish between *M. tuberculosis* complex and nontuberculous mycobacteria. Results were confirmed by real-time polymerase chain reaction (*10*).

Overall, *M. bovis* was isolated from more than one fourth of tissue samples and in 42% of all positive cultures. Significantly more *M. bovis* isolates were obtained from Mbororo zebu (30/75) than from Arab zebu (26/124) ($p = 0.004$). The difference remained significant when the type of condemnation and type of organ were included in a multivariate logistic regression model.

Spoligotyping, as described (*12*), was used as a tool for identifying *M. bovis* within the *M. tuberculosis* complex (lack of spacers 3, 9, 16, and 39–43) but also yielded insights into the epidemiology of *M. bovis*. In total, 12 different spoligotypes were found among the 55 *M. bovis* isolates; 51 (92.7%) of 55 isolates were in 8 clusters (≥2 strains), which showed a homogenous population structure (Figure).

The predominant spoligotype in our study was SP1, with a cluster of 22 strains (40%), as was the case in the study of Cameroon (*7*). SP1 that lacks spacer 30 corresponds to C1; 2 other clusters described in Cameroon (C1 and C5) were also found in Chad (SP2 and SP4). The finding of a high proportion of the same spoligotypes in the 2 countries indicates cross-border movement of cattle. A substantial degree of recent transmission of *M. bovis* strains among cattle is supported by the apparently high prevalence (7%) of TB-like lesions at the slaughterhouse in N'Djaména. However, the homogeneity of bovine strains could also be due to the absence of introduction of new spoligotypes in this particular area. Certain Cameroonian clusters (C7, C8, C9, and C10) (*7*) were only detected in the Adamaoua region, not in northern Cameroon or our Chadian study. The established measures of the Cameroonian government to prevent movement of cattle between the Adamaoua and the 2 northern regions appear effective. As to other neighboring countries, a recent publication describes 15 *M. bovis* isolates from cattle in Nigeria, and these also lack spacer 30 (*13*). This feature seems to be a characteristic of *M. bovis* strains in Central Africa.

Fifteen strains (8 from Arab and 7 from Mbororo zebu) were typed with the IS*6110* restriction fragment length polymorphism (*14*) method, of which 11 and 4 isolates contained 2 or 1 band, respectively (data not shown). Therefore, Chadian *M. bovis* strains belong to low IS*6110* copy number strains. Strains lacking spacer 30 had a band at 1.9 kb, in accordance with the findings in Cameroon (*7*). No association was found between the number of bands and the cattle breed. IS*6110* typing indicated 6 clusters and, thus, was of lower discriminatory power than spoligotyping. In a recent study, variable number of tandem repeat typing was more discriminatory for Chadian *M. bovis* strains than IS*6110* and spoligotyping (*15*).

Conclusions

The first mycobacterial laboratory established in Chad confirmed bovine TB in Chadian herds by culturing and characterizing *M. bovis*. A high ongoing and cross-border transmission of *M. bovis* in cattle is suspected, but further

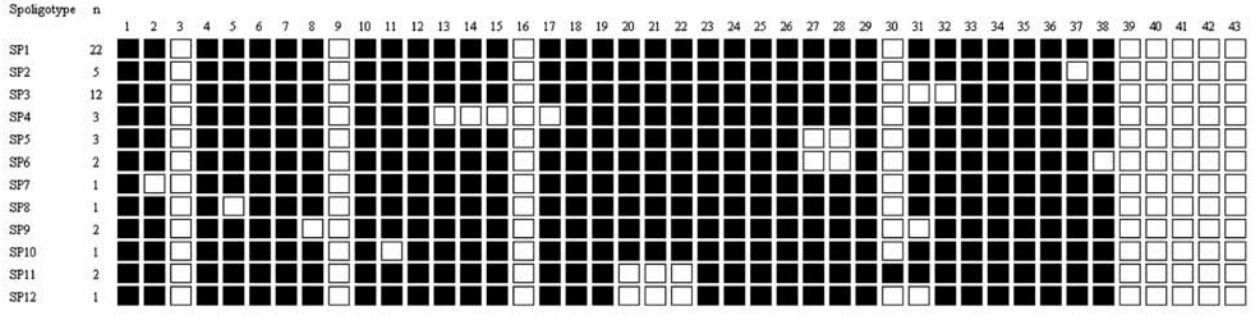

Figure. Spoligotypes obtained from 55 *Mycobacterium bovis* isolates from Chadian zebus.

molecular epidemiology studies are needed to analyze its modes and risk factors. The apparently higher susceptibility of Mbororo zebus to *M. bovis* infection should be followed-up with immunologic assays.

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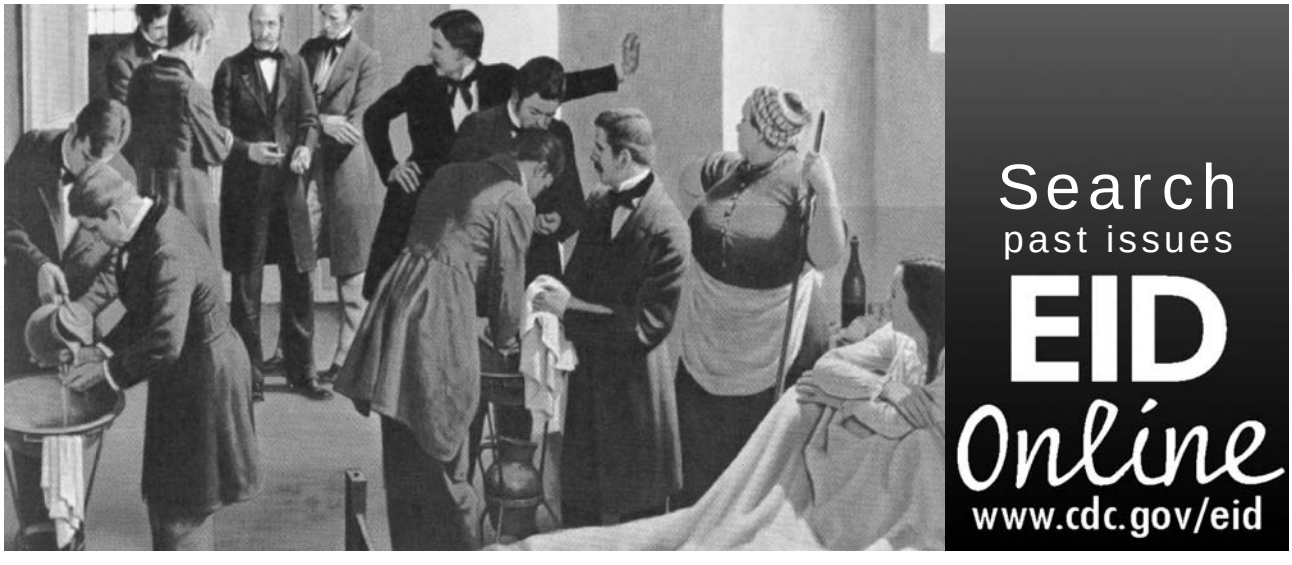

Intact *pks15/1* in Non–W-Beijing *Mycobacterium tuberculosis* Isolates

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To determine whether intact *pks15/1* is unique to the W-Beijing family, we investigated 147 *Mycobacterium tuberculosis* strains with different IS*6110* genotypes. Intact *pks15/1* was found in 87.8% of cerebrospinal fluid and 84.9% of sputum isolates. It was found not only in W-Beijing strains (≈97%) but also in other genotypes (38.5%–100%).

Two structurally related families of cell envelope lipids, phthiocerol diesters and phenolic glycolipids, are virulence factors of *Mycobacterium tuberculosis* and *M. leprae*. They are also produced by other slow-growing species, in particular the pathogenic species *M. marinum*, *M. ulcerans*, and members of *M. tuberculosis* complex (*1*). Phthiocerol diesters are composed of a mixture of long chain β-diols that are esterified by multimethyl-branched fatty acids. Depending on the asymmetric centers bearing the methyl branches (D or L series), the fatty acids are called mycocerosic or phthioceranic acids, respectively, and the corresponding complex lipids are named dimycocerosates of phthiocerol (DIMs) or diphthioceranates of phthiocerol (DIPs) (*1*). The phenolic glycolipids (PGLs) consist of a lipid core similar to those of DIMs or DIPs but ω-terminated by an aromatic nucleus that is glycosylated by type- or species-specific mono-, tri-, or tetrasaccharide. Several lines of evidences suggest that PGLs are involved in the pathogenesis of mycobacterial infections. PGL-1 from *M. leprae* inhibits the proliferation of T lymphocytes after stimulation with concanavalin A (*2*). Moreover, PGL-1 seems to be associated with resistance to intracellular killing by macrophages (*3*) and promotes phagocytosis of *M. leprae* by macrophages and Schwann cells by binding to complement component C3 or laminin α2 chain, respectively (*4*,*5*). Similarly, PGLs produced by a subset of *M. tuberculosis* isolates inhibit the host Th1-type T-cell and cytokine response (*6*). All *M. tuberculosis* strains tested that produce PGLs belong to the W-Beijing family and show a "hypervirulent" phenotype, in comparison with the clinical isolate *M. tuberculosis* CDC1551 and the laboratory strain *M. tuberculosis* H37Rv in the murine model (*6*) and rabbit model of meningitis (*7*).

Previous study identified the involvement of the gene *pks15/1* in the biosynthesis of PGLs; disruption of this gene generated a PGL-deficient mutant (*8*). Sequence alignment of the *pks15/1* gene, when compared to the non–PGL-producing strains, *M. tuberculosis* H37Rv, Erdman, Mt103, and CDC1551, that contain 2 open reading frames [*pks1* (Rv2946c) and *pks1*5 (Rv2947c)], showed a 7-bp insertion in PGL-producing strains *M. tuberculosis* strain 210, belonging to the W-Beijing family, and *M. canetti*, whereas *M. bovis* and *M. bovis* BCG contained only a guanine insertion. This 7-bp or 1-bp insertion causes a frameshift mutation in the *pks1*5, resulting in an intact *pks15/1* with additional codons (*8*). Similar results have been shown in other W-Beijing strains, *M. tuberculosis* HN878, W4, and W10, which contain the 7-bp insertion and produce PGLs (*6*).

In Thailand, the Beijing genotype is the predominant genotype among tuberculosis (TB) patients, particularly in patients with TB meningitis (unpub. data), which suggests recent transmission of this genotype in the country. Similarly, the Beijing genotype has been found frequently in Asia (*9*–*11*). Previous studies have shown that the *M. tuberculosis* strains belonging to this genotype contain an intact *pks15/1* and can produce PGLs that associated with the hypervirulent phenotype (*6*,*7*). The goal of our study was to determine whether the hypervirulence of the W-Beijing strains due to the ability to produce PGLs is unique among this family by investigating the *pks15/1* gene of the Beijing strains compared to other strains that can cause diseases similar to those caused by Beijing strains.

The Study

One hundred forty-seven clinical isolates of *M. tuberculosis* were obtained from the Molecular Mycobacteriology Laboratory, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand, and the T-2 project from 1997 to 2001 (Table). These strains were isolated from 74 cerebrospinal fluid (CSF) samples and 73 sputum samples from 147 different patients. DNA from these isolates was isolated by an enzymatic method and submitted for genotyping by performing the IS*6110* restriction fragment length polymorphism with the standard method (*12*) and for sequencing the *pks15/1* region (*8*).

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Table. Number of *Mycobacterium tuberculosis* genotypes and strains containing an intact *pks15/1**

Genotype	No. strains isolated from CSF	No. CSF strains containing intact *pks15/1* (%)	No. strains isolated from sputum	No. sputum strains containing intact *pks15/1* (%)
Beijing	42	41 (97.6)	31	30 (96.8)
Single-banded	10	8 (80.0)	10	9 (90.0)
2–5 bands	5	4 (80.0)	11	10 (90.9)
Nonthaburi	4	4 (100)	8	8 (100)
Heterogeneous with >5 bands	13	8 (61.5)	13	5 (38.5)
Total	74	65 (87.8)	73	62 (84.9)

*CSF, cerebrospinal fluid.

Using the genotyping results, we categorized *M. tuberculosis* isolates into Beijing, single-banded, few-banded (2–5 bands), Nonthaburi, and heterogeneous with >5 bands (Table and Figure 1), as recently reported (*13*,*14*). All *M. tuberculosis* genotypes were sequenced around the junction of *pks1*5 and *pks1* (corresponding to the *M. tuberculosis* H37Rv sequence) to determine whether they contained an intact *pks15/1* or separated *pks1*5 and *pks1*. Unexpectedly, the results showed that the 7-bp insertion of *pks1*5 that causes a frameshift mutation resulting in an intact *pks15/1* was found in most strains of all genotypes, except the heterogeneous group with >5 bands (Table and Figure 2).

Conclusions

The intact *pks15/1* has been shown to be responsible for the production of phenolic glycolipids and is seemingly found in *M. tuberculosis* W-Beijing family, but it was not found in *M. tuberculosis* CDC1551 and H37Rv (*8*). Previous studies suggested that PGLs produced by the *M. tuberculosis* W-Beijing family were associated with the hypervirulent phenotype by inhibiting the innate immune response (*6*,*7*). The intact *pks15/1* has also been shown to be nonpolymorphic in the W-Beijing family; it was found in all 102 W-Beijing strains tested (*15*). From this observation, we hypothesized that if the ability to produce PGLs is among the factors that make this family more virulent than others, the intact *pks15/1* should be absent in strains other than the W-Beijing family. Our results showed that the 7-bp insertion of the *pks15/1* was not only present in the W-Beijing family but also in other *M. tuberculosis* genotypes. Although almost all Beijing strains contain the intact *pks15/1* (≈97%), 38.5%–100% of strains of other genotypes also contain it. These strains could, therefore, produce PGLs and cause both pulmonary and disseminated diseases as the W-Beijing strains do.

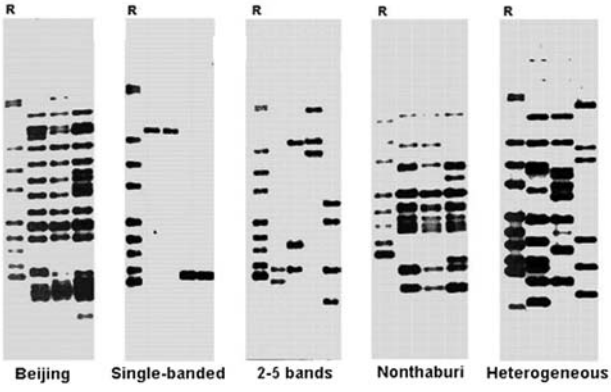

Figure 1. IS*6110* hybridization patterns of each *Mycobacterium tuberculosis* genotype. R indicates the *M. tuberculosis* Mt 14323 strain used as the positive control for IS*6110* typing.

Our results showed no significant difference in the percentage of *M. tuberculosis* isolates with an intact *pks15/1* gene between CSF isolates (65 [87.8%] of 74) and sputum isolates (62 [84.9%] of 73). The hypothesis that the hypervirulence of the W-Beijing family is solely attributable to *pks15/1* is still inconclusive. This family may have only recently been transmitted globally and may have had more

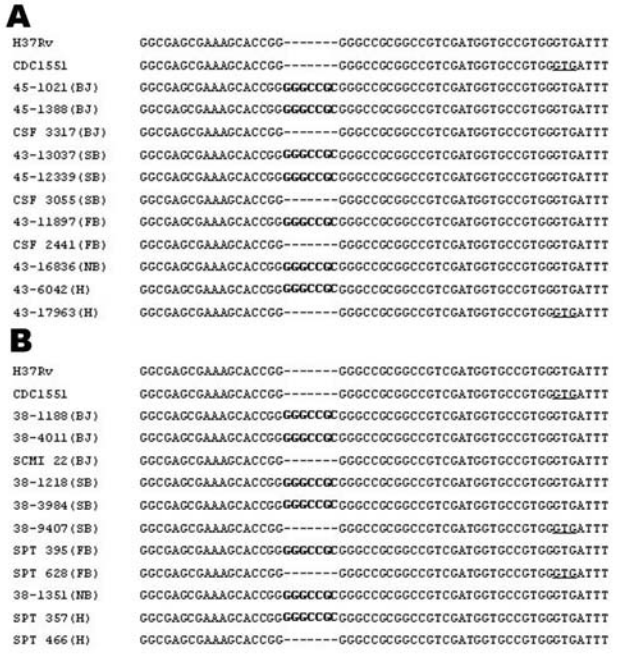

Figure 2. Sequence alignment of region corresponding to the 3 portion of *pks15* and 5′ portion of *pks1* in various *Mycobacterium tuberculosis* genotypes. A) *M. tuberculosis* strains isolated from cerebrospinal fluid. B) *M. tuberculosis* strains isolated from sputum. Letters in brackets refer to IS*6110* restriction fragment length polymorphism patterns: BJ, Beijing; SB, single banded; FB, 2–5 bands; NB, Nonthaburi; H, heterogeneous. The 7-bp insertion is shown in **boldface**, and the start codon of the *pks1* gene is underlined.

chances to cause infections and disease than other families. Although PGLs are involved in the hypervirulence of the PGL-producing strains, they are not a unique characteristic of the W-Beijing family. If W-Beijing strains are more virulent than others, other virulence determinants besides PGLs must be responsible for the hypervirulent phenotype.

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Coronavirus HKU1 Infection in the United States

Frank Esper,* Carla Weibel,† David Ferguson,† Marie L. Landry,† and Jeffrey S. Kahn†

In 2005, a new human coronavirus, HCoV-HKU1, was identified in Hong Kong. We screened respiratory specimens collected from December 16, 2001, to December 15, 2002, from children <5 years of age who tested negative for respiratory syncytial virus, parainfluenza viruses, influenza virus, and adenovirus for HCoV-HKU1 by reverse transcription–polymerase chain reaction. Overall, 1,048 respiratory specimens from 851 children were tested, and 9 HCoV-HKU1–positive children (1%) were identified, 2 of whom had 2 positive specimens. Children who had HCoV-HKU1 infection had evidence of either upper or lower respiratory tract infection or both. Two patients had disease beyond the respiratory tract. HCoV-HKU1 was identified from December 2001 to February 2002. Sequence analyses suggest that a single strain was circulating. HCoV-HKU1 is therefore likely circulating in the United States and is associated with upper and lower respiratory tract disease.

Lower respiratory tract disease accounts for ≈4 million deaths annually worldwide (*1*). Viruses such as influenza virus, respiratory syncytial virus (RSV), and parainfluenza viruses are responsible for much of this respiratory tract infection. However, in a substantial proportion of respiratory tract disease, no pathogen is identified (*2*).

Coronaviruses (CoV) infect a wide variety of mammals and birds, causing disease of the respiratory tract, gastrointestinal tract, and central nervous system. These viruses may be transmitted from species to species (*3*). In humans, CoV have been associated with community-acquired upper respiratory tract infections (*4*). Human CoV (HCoV) have also been implicated in outbreaks of diarrhea as well as in demyelinating disorders of the central nervous system, though these data are controversial (*5*,*6*). The study and identification of HCoV have been hampered by the difficulty in propagating these viruses in vitro.

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The identification of the severe acute respiratory syndrome–associated CoV in 2003 sparked renewed interest in the study of HCoV (*7*), and 4 previously unidentified HCoV have subsequently been discovered. HCoV-NL63, HCoV-NL, and the New Haven coronavirus (HCoV-NH) are closely related group I CoV and likely represent strains of the same species of virus (*8*–*10*). HCoV-NL63 and HCoV-NL were originally identified by cell culture techniques, while HCoV-NH was discovered by using broadly reactive CoV molecular probes. These related viruses were identified in both children and adults with respiratory tract disease. HCoV-NH was found in 8.8% of children <5 years of age whose specimens originally tested negative for RSV, influenza virus, parainfluenza viruses, and adenoviruses (*10*). Furthermore, these newly discovered viruses may be the cause of disease beyond the respiratory tract. In a case-control study, HCoV-NH was found to be associated with Kawasaki disease (*11*), although these data are controversial (*12*,*13*).

In 2005, Woo et al. reported a novel group II CoV, designated HCoV-HKU1, from a 71-year-old man with pneumonia (*14*) who had recently returned to Hong Kong from the Shenzhen, China. As in the discovery of HCoV-NH (*10*), this virus was detected with molecular probes. Although growth of HCoV-HKU1 in multiple cell lines was unsuccessful, the complete genomic sequence was obtained. Phylogenetic analysis showed that this new group II CoV is most closely related to the mouse hepatitis virus and is distinct from HCoV-OC43, the only other known group II HCoV. Screening of 400 nasopharyngeal aspirates by reverse transcription–polymerase chain reaction (RT-PCR) with HCoV-HKU1–specific primers showed 1 other HCoV-HKU1 isolate from a 35-year-old woman with pneumonia. After the original report, HCoV-HKU1 was identified in 10 patients in northern Australia (*15*). Respiratory samples were collected between May and August (winter in Australia) and screened by RT-PCR with both nonspecific CoV and specific HKU1 primers.

Most HCoV-HKU1–positive samples originated from children in the later winter months. However, the seasonal and geographic distribution of this virus is still unclear. To address these issues, we sought to determine whether HCoV-HKU1 circulated in New Haven, Connecticut, and to define clinical characteristics associated with HCoV-HKU1 infection in infants and children.

Methods

Clinical Specimens

Nasopharyngeal swabs and aspirates submitted to the clinical virology laboratory at Yale–New Haven Hospital from December 16, 2001, to December 15, 2002, for respiratory virus diagnosis were initially tested for RSV, parainfluenza viruses (types 1–3), influenza A and B viruses, and adenovirus by direct immunofluorescence assay. Respiratory specimens were screened for human metapneumovirus (*16*) and HCoV-NH (*10*) by RT-PCR. Specimens originated from the emergency department, inpatient wards, intensive care units, and the hospital-affiliated primary care outpatient clinic and were submitted at the discretion of the medical teams. Clinical specimens from children <5 years of age that tested negative by direct immunofluorescence assay were tested for HCoV-HKU1 as described below. Collection of specimens and clinical data was approved by the Yale University Human Investigation Committee and compliant with Health Insurance Portability and Accountability Act regulations.

RT-PCR Screening

RNA from each respiratory specimen was extracted with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Random hexamer primers synthesized by the oligonucleotide laboratory, Department of Pathology, Yale University School of Medicine, were used to create a cDNA library for each specimen. Reverse transcription reactions were performed with MuMLV RT (New England Biolabs, Beverly, MA, USA), according to the manufacturer's specifications. Each cDNA was subsequently screened for the presence of HCoV-HKU1 by polymerase chain reaction with HotStar Taq polymerase (Qiagen), according to the manufacturer's specification. Primers used to screen respiratory specimens were identical to those described by Woo et al. (*14*). The forward primer, 5′ GGTTGGGATTATCCTAAATGTGA, and reverse primer, 5′ CCATCATCACTCAAAATCATCATA, produce an amplicon that corresponds to nucleotides 15409–15848 of the HCoV-HKU1 replicase 1B gene (GenBank accession no. AY597011) and yields an amplicon of 439 bp. Amplification cycles were as follows: 95°C for 15 min; followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and completed with a final extension cycle of 72°C for 10 min. Each set of reverse transcription and polymerase chain reactions contained appropriate negative controls. Sequencing was performed on an Applied Biosystems 3730 XL DNA Analyzer (Foster City, CA, USA) at the W.M. Keck Biotechnology Resource Lab, Yale University School of Medicine.

Clinical Data

Medical records of all HCoV-HKU1–positive children were reviewed. Demographic data, history of illness, and results of clinical examination and laboratory studies were recorded on a standard collection form. The Yale University Human Investigation Committee approved collection of specimens and clinical data.

Results

From December 16, 2001, to December 15, 2002, 1,048 respiratory specimens from 851 children were tested by RT-PCR for HCoV-HKU1. Specimens from 9 of these children (1%) tested positive for HCoV-HKU1. Specimens from these children tested negative for RSV, parainfluenza viruses (types 1–3), influenza A and B viruses, and adenovirus by direct immunofluorescence assay as well as human metapneumovirus and HCoV-NH by RT-PCR. Two children had 2 specimens that tested positive for HCoV-HKU1. For each of these 2 children, the positive specimens were collected <10 days apart. Children whose specimens tested positive for HCoV-HKU1 infection had clinical evidence of either upper or lower respiratory tract infection or both (Table). The most common clinical findings were rhinorrhea (100%), cough (67%), fever (67%), and abnormal breath sounds on auscultation (44%). Hypoxia (oxygen saturation of <90%) was observed in only 1 patient. Chest radiographs were obtained for 4 patients, all of whom had abnormal findings that included peribronchial cuffing, atelectasis, hyperinflation, or infiltrates. One patient (patient 3) had respiratory decompensation requiring ventilatory support and was admitted to the pediatric intensive care unit. This patient had no history of underlying illness, had not been premature, and was 1 month of age at the time of specimen collection.

Two patients had evidence of disease beyond the respiratory tract. One patient (patient 1) was hospitalized for new-onset seizures. Workup for a central nervous system infection, including a lumbar puncture and head magnetic resonance imaging, was unrevealing. Although a febrile seizure remains a possible diagnosis, no evidence of fever was reported by the mother or noted during the hospital stay. A second patient (patient 7) was hospitalized with hepatitis. This patient had undergone liver transplantation 3 months before admission. Immunosuppressive medications included tacrolimus and prednisolone. The patient

Table. Clinical manifestations associated with human coronavirus HKU1 infection*

Patient no. (sex)	Age (mo)	Specimen collection date	Length of hospitalization (d)	Diagnosis	Underlying illness	Signs/symptoms	Chest radiographic findings
1 (M)	4	Dec 18	1	New onset seizures	RAD	Rhinorrhea, cough	Not obtained
2 (M)	12	Dec 20	4	Pneumonia	Cystic fibrosis	Fever, rhinorrhea, cough, wheezing, rhonchi, retractions, rash	Infiltrates
3 (F)†‡	1	Dec 22, 31	9	Pneumonia	None	Fever, hypoxia, rhinorrhea, cough, wheezing, rhonchi, retractions, nasal flaring, apnea	Hyperinflation, infiltrates, peribronchial cuffing, atelectasis
4 (M)	2	Jan 5	NH	Fever	None	Fever, rhinorrhea	Not obtained
5 (F)†	20	Jan 14, 16	2	ALTE	None	Fever, rhinorrhea, cough	Infiltrates, atelectasis
6 (F)	3	Jan 16	NH	Bronchiolitis	Prematurity (34 weeks)	Rhinorrhea, cough, wheezing, rhonchi	Peribronchial cuffing
7 (M)	13	Jan 19	10	Hepatitis	Biliary atresia, liver transplantation	Rhinorrhea, rhonchi, abnormal LFT results	Not obtained
8 (F)	16	Jan 19	1	Fever	Sickle cell anemia	Fever, rhinorrhea	Not obtained
9 (F)	13	Feb 1	NH	Fever	None	Fever, rhinorrhea, cough	Not obtained

*M, male; F, female; RAD, reactive airway disease; NH, not hospitalized; ALTE, apparent life-threatening event; LFT, liver function tests (aspartate aminotransferase 238 U/mL, alanine aminotransferase 373 U/mL, alkaline phosphatase 406 U/mL, bilirubin [total/direct] 0.15/0.05 mg/dL).
†Two respiratory specimens tested positive for human coronavirus HKU1.
‡Patient required mechanical ventilation and admission to the pediatric intensive care unit.

was also receiving ganciclovir for cytomegalovirus prophylaxis. The onset of abnormal liver enzyme levels occurred several days after the onset of respiratory symptoms and after collection of the respiratory specimen that tested positive for HCoV-HKU1. No evidence of abnormal liver function was detected (both prothrombin time and partial thromboplastin time were within normal ranges). Serologic assays for hepatitis viruses A, B, and C were negative. A liver biopsy specimen did not show evidence of rejection. Levels of the serum liver enzymes slowly decreased during hospitalization. No interventions (e.g., changes in immunosuppressive therapy) were performed.

All HCoV-HKU1 infections occurred during a 7-week period from December 2001 to February 2002 (Figure). HCoV-HKU1–positive samples accounted for 5% of samples screened during that period. No HCoV-HKU1–positive isolates were detected in specimens collected in the remainder of the study period.

The RT-PCR amplicon from each positive specimen was sequenced. Nucleotide and amino acid identity between replicase 1B region of the original HCoV-HKU1 isolate and the New Haven isolates were both >95%. Rare polymorphisms (<1% of sequence) were noted in the HCoV-HKU1 sequences of the New Haven isolates (data not shown), which suggests that a single strain was circulating in the community during the study period.

Discussion

We report the first identification of HCoV-HKU1 in the Western Hemisphere. These findings suggest that HCoV-HKU1 may have a worldwide distribution. We detected this coronavirus in 1% of children screened. All HCoV-HKU1–positive samples tested negative for RSV, influenza virus, parainfluenza viruses, adenoviruses, HCoV-NH, and human metapneumovirus. Our laboratory did not have access to materials from Hong Kong; therefore, the results cannot represent laboratory contamination from material obtained elsewhere. The percentage of positive specimens was similar to that described by Woo et al. (1 [0.25%] of 400) (*14)* and Sloots et al. (10 [3.1%] of 324) (*15*), which suggests that infection with HCoV-HKU1 may be uncommon or that the virus has properties that decreases the likelihood of detection, such as a brief period of viral shedding. Our study, the study by Sloots et al., and the original study by Woo et al. screened respiratory specimens submitted to a diagnostic laboratory. Therefore, HKU1 may be a common virus that causes symptomatic disease in only a relatively small percentage of infected persons. All HCoV-HKU1–positive specimens were collected from December 2001 to February 2002, which implies a winter distribution. The study by Sloots et al. also detected HCoV-HKU1 predominantly in the winter, although only respiratory samples submitted during winter months were screened. Whether the seasonal distribution of HCoV-HKU1 varies from year to year is not known.

Similar to the patients described by Woo et al., several HCoV-HKU1–positive patients had evidence of lower respiratory tract involvement (2 patients with pneumonia and 1 patient with bronchiolitis). Two of these patients had underlying illness. However, most patients identified in our study had only mild upper respiratory tract symptoms. Most HCoV-HKU1 infections in children, similar to other

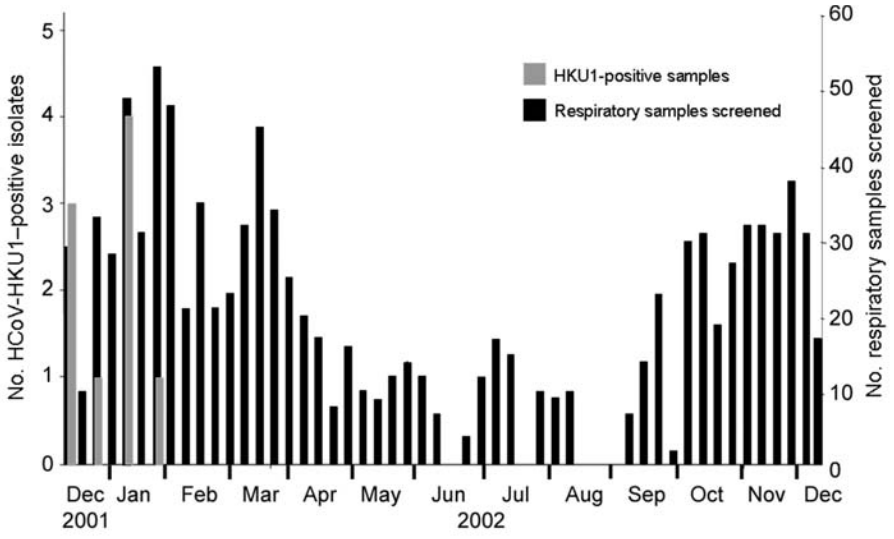

Figure. Weekly distribution of human coronavirus (HCoV)-HKU1 infection in children <5 years of age, December 16, 2001, to December 15, 2002, New Haven, Connecticut. The weekly distributions of HCoV-HKU1 isolates are shown as gray bars (left axis). The total number of samples collected by week are indicated by black bars (right axis).

common HCoV infections, likely result in mild disease (*4*). The Australian study did not perform a detailed clinical review of HCoV-HKU1–positive patients, but the authors note that symptoms are consistent with those of acute respiratory tract illness (*15*).The severity of disease caused by SARS-CoV in children was also relatively mild for reasons that are not yet understood (*17*). Underlying illness and preexisting lung disease may predispose to a more severe clinical course.

Evidence of hepatitis in 1 child who tested positive for HCoV-HKU1 is an intriguing finding. HCoV-HKU1 is most closely related to the murine hepatitis virus, a virus that causes hepatitis as well as demyelinating disease in mice (*18*). Because of this patient's medical history (liver transplantation) and compromised immune status, many potential causes of hepatitis exist, though serologic assays and liver biopsy findings were unrevealing. Several reports have found coronavirus-like particles in stool of persons with gastrointestinal disease (*19*), which suggests that these viruses, like coronaviruses of animals, can cause disease of the gastrointestinal tract. Future studies will be needed to determine whether HCoV-HKU1, or other common human coronaviruses, play a role in liver disease.

Our study had several shortcomings. We limited our screening to respiratory specimens that were collected at the discretion of the medical team, we did not include a control group of asymptomatic children, and serum samples were not available for serologic assays. Nonetheless, our findings show that HCoV-HKU1 is circulating in New Haven, Connecticut, and is associated with both upper and lower respiratory tract disease and perhaps extrapulmonary disease.

The genetic variability of HCoV-HKU1 is unknown. The study by Sloots et al. suggests 2 genotypes when comparing the Australian isolates to the prototype Hong Kong strain (*15*). If multiple genotypes exist, they may not all be detected with the primer set used. This limitation would result in an underestimation of this virus in our study. However, the region of the replicase 1B gene targeted by the primers used (*14*) is highly conserved among other coronaviruses, and our screening was unlikely to have lacked sensitivity for that reason. Also, only rare polymorphisms were detected on the sequence analysis of the 9 individual isolates, which suggests that this region is highly conserved. However, to establish the true prevalence of HKU1, use of primers with known specificity and sensitivity for HCoV-HKU1 will be critical.

In conclusion, we show that HCoV-HKU1 circulates in the United States, and the strain identified in New Haven is similar to the original strain described from Hong Kong. Whether this newly recognized pathogen is responsible for a substantial proportion of respiratory tract disease in children remains to be determined. Future studies are required to determine the epidemiologic features and clinical spectrum of this newly recognized pathogen.

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Shiga-toxigenic *Escherichia coli* O157 in Agricultural Fair Livestock, United States

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Agricultural fairs exhibiting livestock are increasingly implicated in human Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157:H7) outbreaks. To estimate livestock STEC O157:H7 prevalence at US fairs, we collected 2,919 fecal specimens at 29 county fairs in 2 states and at 3 state fairs in 2002. Fly pools were also collected. STEC O157:H7 was isolated from livestock at 31 (96.9%) of 32 fairs, including 11.4% of 1,407 cattle, 1.2% of 1,102 swine, 3.6% of 364 sheep and goats, and 5.2% of 154 fly pools. Cattle, swine, and flies at some fairs shared indistinguishable STEC O157:H7 isolate subtypes. In 2003, a total of 689 ambient environmental samples were collected at 20 fairgrounds 10–11 months after 2002 livestock sampling while fairgrounds were livestock-free. Four beef barn environmental samples at 3 fairgrounds yielded STEC O157:H7. These data suggest that STEC O157 is common and transmissible among livestock displayed at agricultural fairs and persists in the environment after the fair.

Each year, ≈3,500 state and county fairs in the United States attract >125 million urban, suburban, and rural visitors (*1*). Livestock exhibits, which are popular and common at most of these fairs, provide an opportunity for both direct and indirect human contact with animals that may be subclinically infected with zoonotic enteric pathogens.

Fair attendance increases infection risk for human Shiga-toxigenic *Escherichia coli* O157:H7 (STEC O157) in the United States (*2*). Since 1999, at least 7 US human STEC O157 outbreaks have been associated with visits to agricultural fairs displaying livestock, resulting in thousands of illnesses, >300 culture-confirmed infections, at least 36 cases of hemolytic uremic syndrome, and 2 deaths (*1*,*3*,*4*). Fair STEC O157 outbreaks in the United States have been associated with ruminant contact, contaminated water, and contact with animal environments (*2*,*5*,*6*).

The objectives of this study were to estimate fecal STEC O157:H7 prevalence in livestock on display at US agricultural fairs and to estimate STEC O157:H7 prevalence in the postfair environment, 10–11 months later, when animals were absent. Because pest flies may act as vectors of STEC O157:H7 (*7*) and are abundant at fairs, we also estimated STEC O157:H7 prevalence in flies at fairs. Finally, we compared clonality and estimated diversity of STEC O157:H7 isolates from animals and flies at fairs and from the postfair environment.

Methods

Fecal and Fly Sampling

We collected fresh fecal specimens at 32 agricultural fairs at 29 small or local county fairs in 2 midwestern states and at 3 large state fairs in 2 midwestern states and 1 southern state. County fair fecal sampling targeted 25 cattle and 25 pigs. State fair fecal sampling targeted 60–70 each for market and breeding beef, market and breeding swine, and dairy cattle. Other livestock fecal specimens (e.g., sheep, goats, equids, and poultry) were collected as available. To maximize the likely number of source farms per fair from which samples originated, we obtained 1 fecal specimen per cow or 1 fecal specimen per pen for animals displayed in small groups (pigs, sheep, goats, and poultry) with a common owner. If present, adult muscoid pest flies (house flies, *Musca domestica*; stable flies, *Stomoxys calcitrans*; and blow flies, *Calliphoridae* sp.) were trapped live with fly pheromone-baited jug traps or live-netted from livestock buildings, dumpsters, garbage cans, feed containers,

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and animal wash stations. Fecal and fly samples were collected in summer and early fall of 2002 while fairs were open to the public. Permission to collect samples at fairs was obtained in advance from fair boards or fair managers and, in some cases, from individual animal owners. Fair, animal owner, and animal anonymity were maintained as a condition of permission to sample.

Environmental Sampling

We collected fairground environment samples from 19 county fairgrounds in 2 states and 1 state fairground in the summer of 2003 from among the 32 fairs visited for livestock sampling in 2002. At the time of environmental sampling, none of the fairgrounds had any livestock; the 19 county fairgrounds (but not the state fairground) had had no or very limited livestock on the premises since the fair in the previous year. Environmental sampling in 2003 was conducted 10–11 months after the 2002 livestock sampling at each fair. Samples collected included soil, bedding (sand, sawdust, woodchips), pest flies, dried manure, standing water, and surface swabs of concrete, wood, and metal structures such as floors, walls, and railings.

County fairground environmental sampling consisted of ≥30 samples per fairground, 10 each from cattle, swine, and show arena areas. Of each set of 10, a total of 5 were collected at ground level, and 5 were collected from above-ground surfaces. For the state fairground, 25 samples were collected each from the cattle, swine, and show arena areas. Of each set of 25, a total of 10 were from the ground level, 10 were from above-ground surfaces, and 5 were from ceilings.

Isolation, Serotyping, and Characterization of STEC O157

Fecal enrichment was performed as previously described in gram-negative broth containing vancomycin (8 mg/L), cefixime (0.05 mg/L), and cefsulodin (10 mg/L) (GN-VCC) for 6 h at 37°C, followed by immunomagnetic separation (IMS) (*8–10*). Bead IMS aliquots were spread plated onto ChromAgar O157 (CHROMagar, Paris, France) containing 0.63 mg/L potassium tellurite (1× tellurite ChromAgar O157 [TCA]), except for IMS beads derived from enriched swine feces, which were plated onto ChromAgar O157 containing twice (1.25 mg/L) the potassium tellurite (2× TCA). Live-trapped, adult pest fly pools were chilled at –20°C until immobile but viable, counted, speciated, placed into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA), and crushed with a solid glass rod. Fly broth was enriched by adding either 2 mL of 1.5× (60 g/L) brilliant green bile broth (BGB) or GN-VCC per 5 flies added directly to the Whirl-Pak bags in which the flies were crushed. The bags were incubated for 6 h at 37°C, analyzed by IMS, and plated on 1× TCA. Environmental samples were enriched in 1.5× BGB for 6 h at 37°C, analyzed by IMS, and plated on 1× TCA (*4*). Up to 5 mauve-pink STEC O157 colonies per 1× or 2× TCA plate were serotyped by enzyme immunoassay using monoclonal antibodies to *E. coli* O157 and *E. coli* H7 (*8*) and analyzed by polymerase chain reaction (PCR) assays for *stx*1, *stx*2 (Shiga toxin), *eae* (intimin), rfb_{O157} (O157 O-antigen), and $fliC_{H7}$ (H7 flagellum) genes (*11*,*12*). Depending on the O:H antigens and gene subsets present, individual fecal, fly, or environmental isolates identified as *E. coli* O157 were classified as STEC O157:H7, Shiga-toxin gene PCR-negative (*stx*-negative) *E. coli* O157:H7, or *stx*-negative *E. coli* O157:non-H7.

Pulsed-Field Gel Electrophoresis and Strain Diversity

We conducted pulsed-field gel electrophoresis (PFGE) on representative subsets of fecal, fly, and environmental STEC O157:H7 isolates by using the PulseNet protocol and the restriction endonuclease *Xba*I (*13*). After determining the number and uniqueness of each PFGE pattern for isolates at each fair, we calculated the Simpson diversity index (D) as a measure of within-fair isolate diversity (*14*,*15*). The Simpson D ranges from 0 to 1; higher values represent greater strain diversity. In this case, the Simpson D was the probability that any 2 randomly selected isolates from a given fair had a different (unique) PFGE pattern.

Results

Prevalence of *E. coli* O157 in Fair Animal Feces and Pest Flies

A total of 2,919 livestock fecal samples were collected at 32 fairs, of which 187 (6.4%) were STEC O157:H7 positive. Species-specific STEC O157:H7, *stx*-negative *E. coli* O157:H7, and *stx*-negative *E. coli* O157:non-H7 fecal isolation rates are shown in Table 1. STEC O157:H7 was most prevalent in cattle feces (11.4% of 1,407 beef and dairy cattle). Fair-specific sampling intensities and *E. coli* O157 isolation rates for cattle and swine are shown in the Figure. STEC O157:H7 prevalence at fairs was 0%–36% in cattle and 0%–8% in swine. We commonly isolated *stx*-negative *E. coli* O157:H7 and *stx*-negative *E. coli* O157:non-H7 from cattle and swine, as shown in the Figure. Flies live-trapped at 21 fairs generated 154 fly pools (63 stable fly, 54 house fly, and 37 blow fly). STEC O157:H7 was isolated from 8 (5.2%) pools (7 house fly and 1 blow fly) at 4 fairs. STEC O157:H7-positive fly pools originated from beef barns (6 pools), a swine barn, and an outdoor manure pile. We isolated STEC O157:H7 from 7 of 87 fly pools enriched in 1.5× BGB and 1 of 67 fly pools enriched in GN-VCC.

STEC O157:H7 was isolated from ≥1 livestock species at 28 of 29 county fairs and all 3 state fairs (fair-level

Table 1. *Escherichia coli* O157 livestock fecal or pest fly isolation rates from 32 US county and state fairs, 2002*

Sample	No. samples collected	No. (%) STEC O157:H7 positive	No. (%) *stx*-negative *E. coli* O157:H7 positive	No. (%) *stx*-negative *E. coli* O157:non-H7 positive
Beef cattle	1,163	151 (13.0)	9 (0.8)	7 (0.6)
Dairy cattle	244	10 (4.1)	0	11 (4.5)
Pigs	1,102	13 (1.2)	9 (0.8)	19 (1.7)
Sheep	251	11 (4.4)	0	6 (2.4)
Goats	113	2 (1.8)	0	1 (0.9)
Other livestock†	46	0	0	0
Fly pools	154	8 (5.2)	1 (0.7)	7 (4.6)
All samples	3,073	195 (6.3)	19 (0.6)	51 (1.7)
All livestock	2,919	187 (6.4)	18 (0.6)	44 (1.5)

*STEC, Shiga-toxigenic *Escherichia coli*.
†Includes 15 chickens, 14 rabbits, 10 horses, 2 alpacas, 1 llama, 1 donkey, 1 pony, 1 turkey, and 1 guinea fowl sampled at 8 county fairs.

prevalence 96.9%). Cattle and swine feces were collected at all fairs, while other livestock were variably present for sampling. Flies were unavailable for sampling at 11 fairs primarily because of inclement weather. The fair-level STEC O157:H7 prevalence by species (i.e., number of fairs with STEC O157:H7 present in the species/number of fairs with this species present) was beef cattle, 30/32 (93.8%); dairy cattle, 4/5 (80.0%); pigs, 11/32 (34.4%); sheep, 6/12 (50.0%); goats, 1/5 (20.0%); other livestock, 0/8 (0%); and pest flies, 4/21 (19.0%).

E. coli O157 negative for *stx* was prevalent in fair livestock and flies and was found at 19 of the 32 fairs. *E. coli* O157:H7 negative for *stx* was isolated from 19 samples at 13 fairs (9 beef cattle, 9 pigs, and 1 fly pool). *E. coli* O157:non-H7 negative for *stx* was found in 51 samples at 12 fairs (7 beef cattle, 11 dairy cattle, 19 pigs, 6 sheep, 1 goat, and 7 fly pools).

Prevalence of Postfair Environmental *E. coli* O157

Of 689 environmental samples collected at 20 fairgrounds in the summer of 2003, STEC O157:H7 was isolated from 4 (0.6%) samples at 3 fairgrounds (1 state fairground and 2 county fairgrounds, 15% of sampled fairgrounds). All 4 STEC O157:H7–positive samples were from beef barn environments: 2 dirt samples, 1 house fly pool, and 1 above-ground surface swab. One *stx*-negative *E. coli* O157:H7 and 3 *stx*-negative *E. coli* O157:non-H7 were isolated from beef barn samples at 2 county fairgrounds.

Isolate Characterization and PFGE Patterns

Shiga-toxin and intimin gene profiles of 214 livestock and pest fly *E. coli* O157:H7 isolates are shown in Table 2. Most of these isolates (90.7%) had *stx*2 alone or in combination with *stx*1. Among the 195 (187 livestock feces and 8 pest fly) STEC O157:H7 isolates, all were *eae*-positive except for 1 pig isolate. Among the 4 environmental STEC O157:H7 isolates, 3 were *stx*1 positive and 1 was *stx*-1, *stx*-2 positive; all 4 were *eae*-positive.

PFGE was conducted on a subset of 79 fecal, fly, and environmental STEC O157:H7 isolates, including all isolates derived from 1 state fair and 6 county fairs. PFGE results and Simpson D for each fair's isolates are summarized in Table 3. Diverse PFGE patterns were present at most fairs. We found 47 unique PFGE patterns among the 79 tested isolates from 7 fairs. In 2 instances, STEC O157:H7 PFGE patterns were indistinguishable in bovine and fly isolates from the same fair. In another instance, indistinguishable PFGE patterns were found in cattle, swine, and fly isolates at the same fair. Fly STEC O157:H7 isolates from 1 fair had multiple PFGE patterns. At the 3 fairs where we obtained postfair environmental STEC O157:H7 isolates, no environmental isolate PFGE patterns matched any fecal or fly isolates.

Discussion

The primary aim of the study was to estimate the fecal STEC O157:H7 prevalence in livestock at agricultural fairs across multiple species, multiple fairs, and multiple states to better understand and manage the zoonotic risk. Our data indicate that STEC O157:H7 is endemic and common in ruminant livestock, especially cattle, exhibited at fairs. In addition, swine and pest flies, particularly house flies, may also be infected by or contaminated with STEC O157:H7, albeit at lower prevalence than in cattle. The STEC O157:H7 fecal prevalence of 13% in beef cattle at US fairs in this study (Table 1) is comparable to the STEC O157:H7 fecal prevalence of 13% in summer feedlot cattle (*16*) but less than the STEC O157:H7 fecal prevalence of 28% in feedlot cattle presented for summer slaughter at midwestern meat processing plants (*9*). The STEC O157:H7 fecal prevalence in swine at fairs in this study (1.2%) is similar to the STEC O157 prevalence of 2.0% reported for 350 US swine colon samples collected at slaughter (*17*). We isolated STEC O157 from flies at 19.1% of the 21 fairs from which they were trapped. Data on fly STEC O157 prevalence in livestock settings are limited, but STEC O157 has been isolated from adult house flies on cattle farms (*18,19*). Although comparing fair STEC O157:H7 prevalence across states was not a study objective, we found no difference in either cattle or swine

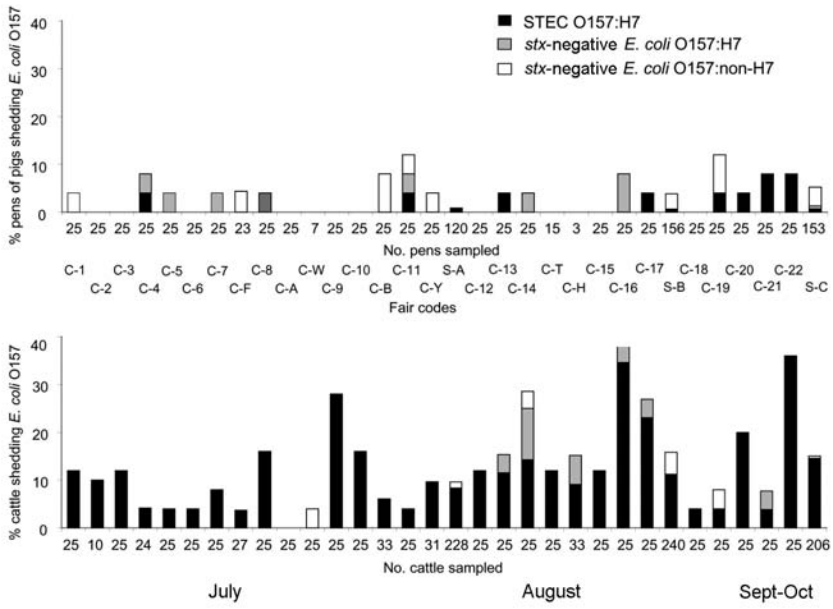

Figure. Fecal prevalence of Shiga-toxigenic (*stx*) *Escherichia coli* (STEC) O157:H7, *stx*-negative *E. coli* O157:H7, and *stx*-negative *E. coli* O157:non-H7 in 1,102 pens of pigs and 1,407 cattle (244 dairy cattle and 1,163 beef cattle) during exhibitions at 3 US state fairs and 29 county fairs, 2002. C, county; S, state. Data are presented in the order that fairs were sampled.

fecal prevalence at fairs in the 3 states surveyed (data not shown).

Study of STEC O157:H7 prevalence at agricultural fairs to some degree represents a "natural experiment" opportunity to investigate the role that animal and environmental hygiene may play in the epidemiology and control of this zoonotic agent in livestock, i.e., to evaluate the "hygiene hypothesis." Husbandry and management practices for US fair animals differ a great deal from those used for commercial livestock. Animal density is high, and exposure to feces is constant in most US commercial settings where livestock are managed for meat, milk, or fiber production. In contrast, animals raised for show competitions at fairs are typically reared individually or in small groups and are meticulously groomed and individually fed (*20*,*21*). Fair livestock are thoroughly washed and cleaned at least daily for several weeks before and especially during fairs. Exhibitors of livestock are typically diligent about maintaining clean, manure-free stalls because hygiene is a judged outcome in competitions. We hypothesized that this emphasis on animal and environmental hygiene would result in a lower STEC O157 prevalence in fair animals compared with commercially reared livestock. Our data, however, indicate that STEC O157:H7 fecal prevalence in fair animals is similar to that of their commercial counterparts. This finding suggests that preharvest efforts to limit STEC O157:H7 prevalence or control transmission in production livestock based solely on cleaning of animals or their environment are unlikely to be successful.

Our finding that fair livestock STEC O157:H7 prevalence was high and comparable to that in production livestock is important for agriculture and public health officials, fair managers, and fair visitors to consider. In contrast to livestock production settings, where only small numbers of people and few children have animal access and contact, livestock exhibits at fairs attract millions of

Table 2. Gene profiles of *Escherichia coli* O157:H7 from livestock fecal and pest fly samples collected at 32 state and county fairs by source species, 2002*

Sample	No. samples collected	No. (%) *E. coli* O157:H7-positive samples	Gene profile, no. isolates (%)				
			*stx*1 only	*stx*2 only	Both *stx*1 and *stx*2	Neither *stx*1 nor *stx*2	*eae*-positive
Beef cattle	1,163	160 (13.8)	0	99 (61.9)	52 (32.5)	9 (5.6)	158 (98.8)†
Dairy cattle	244	10 (4.1)	1 (10.0)	8 (80.0)	1 (10.0)	0	10 (100)
Pigs	1,102	22 (2.0)	0	6 (27.3)‡	7 (31.8)	9 (40.9)	15 (68.2)§
Sheep	251	11 (4.4)	0	5 (45.5)	6 (54.6)	0	11 (100)
Goats	113	2 (1.9)	0	2 (100.0)	0	0	2 (100.0)
Other livestock	46	0	NA	NA	NA	NA	NA
Fly pools	154	9 (5.8)	0	6 (66.7)	2 (22.2)	1 (11.1)	9 (100.0)
All sources	3,073	214 (6.9)	1 (0.5)	126 (58.9)	68 (31.8)	19 (8.9)	205 (95.8)

*Identified by polymerase chain reaction. *stx*, Shiga toxin; *eae*, intimin. NA, not available.
†Both beef cattle *eae*-negative isolates were also *stx*-negative.
‡Includes 1 *eae*-negative swine isolate.
§Includes all 13 *stx*-positive swine isolates and 2 *stx*-negative isolates.

Table 3. PFGE patterns (n = 47) of 79 STEC O157:H7 from animal fecal, fly, and environmental samples at 7 selected state and county fairs, 2002 (livestock and fly isolates) and 2003 (environmental isolates)*

Fair code	Total isolates	PFGE patterns	Unique *Xba*I patterns (P1–P47) (sample type† - no. isolates)	Simpson D (95% CI)‡
SF2	35	25	P1 (F-5, B-2), P2 (B-2), P3 (B-2), P4 (B-2), P5 (B-2), P6–P25§	0.96 (0.92–0.99)
CF19	4	2	P26 (B-1, P-1), P27 (E-2)	0.67 (0.49–0.85)
CF20	9	5	P28 (P-1, B-1, F-1), P29 (F-3), P30 (D-1)¶, P31 (B-1), P32 (D-1)	0.83 (0.73–0.94)
CF22	14	6	P33 (B-5, P-2), P34 (S-3), P30 (D-1)¶, P35 (B-1), P36 (B-1), P37 (B-1)	0.74 (0.59–0.89)
CFB	4	4	P38 (Ef-1), P39 (B-1), P40 (B-1), P41 (S-1)	1.00 (NA)
CFY	4	4	P42 (B-1), P43 (B-1), P44 (B-1), P45 (S-1)	1.00 (NA)
CF16	9	2	P46 (B-8)#, P47 (B-1)	0.22 (0.00–0.57)

*PFGE, pulsed-field gel electrophoresis; STEC, Shiga-toxigenic *Escherichia coli*.
†F, fly (pooled fly sample collected when livestock were present on fair grounds); B, beef cattle; P, pig; E, environment; D, dairy cattle; S, sheep; Ef, environment fly (pooled fly sample collected when fair grounds were closed to the public and no animals were present).
‡Probability that any 2 randomly selected isolates from a given fair have unique PFGE patterns. CI, confidence interval; NA, not available.
§Twenty isolates from SF2 each with a unique PFGE pattern from the following samples: 16 beef cattle, 2 sheep, 1 pig, and 1 dairy cow.
¶Isolates from dairy samples at CF20 and CF22 were indistinguishable; fairs were geographically close to each other.
#A stool STEC O157 isolate from a human clinical case-patient (hemorrhagic colitis) who had visited this fair also had this PFGE pattern.

persons, many of them children. Monitored and controlled human-livestock interaction and contact at fairs is sometimes encouraged. For example, we isolated STEC O157:H7 from feces of a demonstration milk cow at 1 surveyed state fair. Children were encouraged to milk this cow by hand, so this dairy cow had direct contact with hundreds of children each day. Cattle that are fecal-shedding STEC O157:H7 may have concomitant hide contamination at multiple locations and may also shed the pathogen orally (*10*). Many persons likely had contact with this animal's hide. However, to our knowledge, no human STEC O157:H7 infections were associated with this dairy cow. In contrast, a person with hemorrhagic colitis who visited 1 surveyed county fair was infected with an STEC O157:H7 clone that was indistinguishable from multiple fecal isolates found in cattle at that fair (Table 3).

We isolated *E. coli* O157 that did not carry Shiga-toxin genes at several fairs from cattle, pigs, sheep, goats, flies, and the fairground environment (Tables 1 and 2, Figure). This finding underscores the importance of thorough characterization of *E. coli* O157 isolates because not all *E. coli* O157:H7 are STEC O157:H7 and not all *E. coli* O157 have the H7 flagellum antigen or corresponding $flic_{H7}$ gene. The potential of these isolates to cause human disease is unclear. However, nontoxigenic *E. coli* O157:H7/H-negative strains have been associated with sporadic cases and outbreaks of human disease, including hemolytic uremic syndrome, in Europe (*22*,*23*). In addition, *E. coli* O157 Shiga-toxin loss during human infection and during in vitro cultivation are documented (*24*,*25*).

Diverse STEC O157:H7 PFGE subtypes were present at most fairs. This finding is not unexpected because fairs represent a temporary (3–14 days) assemblage of animals from many source farms, STEC O157 is endemic in US livestock (*9*), and livestock STEC O157 clones are diverse between source farms (*26*). Individual animals were not tracked in this study. Therefore, when indistinguishable STEC O157:H7 PGFE subtypes occurred at the same fairs, we could not tell if matches were linked to animals from the same farm, if the same subtype occurred simultaneously on 2 geographically isolated farms, or if the clone was transmitted between animals at the fair. The fact that we observed 2 fairs with indistinguishable fly-livestock isolate PFGE patterns suggests that flies, especially house flies, may be local transmission vectors of STEC O157:H7. Kobayashi et al. (*7*) found STEC O157 in adult house fly intestines and showed experimental shedding by flies for up to 3 days postchallenge. Alternatively, flies and animals may have acquired STEC O157 from a common agricultural fair environmental reservoir such as feed, water, or manure. However, a role for pest flies in the transmission of enteric pathogens to humans is plausible (*27*).

STEC O157:H7 was recovered from the 3 animal-free fairground environments. Environmental STEC O157:H7 may represent residual contamination from previous fairs or other animal events. This environmental contamination may be both an animal biosecurity and a zoonotic risk as a potential source of infection to arriving animals or visiting persons, respectively, at future fair events. PFGE analysis of animal isolates from 2002 and environmental isolates from the same fairs in 2003 did not show any shared STEC O157:H7 isolate subtypes. However, considering the broad observed diversity of STEC O157:H7 clones isolated from fair animals and the limited number of samples that we tested by PFGE, the absence of matching PFGE patterns might be expected.

STEC O157:H7 is a substantial public health risk at fairs. STEC O157:H7 is a frequent infecting or contaminating zoonotic pathogen of animals displayed at agricultural fairs and, to a lesser degree, at the animal-free postfair agricultural environment. Given the high prevalence of STEC O157:H7 at fairs, high fecal prevalence in individual cows, many thousands of exhibited livestock at thousands of agricultural fairs, and millions of human

visitors to fairs each year, fair-associated human STEC O157:H7 outbreaks might be expected to be more frequent. Fortunately, however, STEC O157:H7 zoonotic transmission from livestock to humans, at least in a clinically overt manner, is relatively rare. Our PFGE data showing that cattle and pigs (that were exhibited in different buildings at these fairs) shared indistinguishable STEC O157:H7 subtypes are compatible with intrafair transmission among livestock. Possible vehicles include STEC O157:H7-contaminated pest flies or fomite (e.g., feed, water, shared equipment) exposures. Similarly, humans may also be cross-infected with STEC O157:H7 by livestock or flies at fairs. Recently published guidelines on human interactions with livestock in public settings (*28*,*29*) provide a baseline for developing strategies to lower the zoonotic risk for human STEC O157:H7 infection at agricultural fairs.

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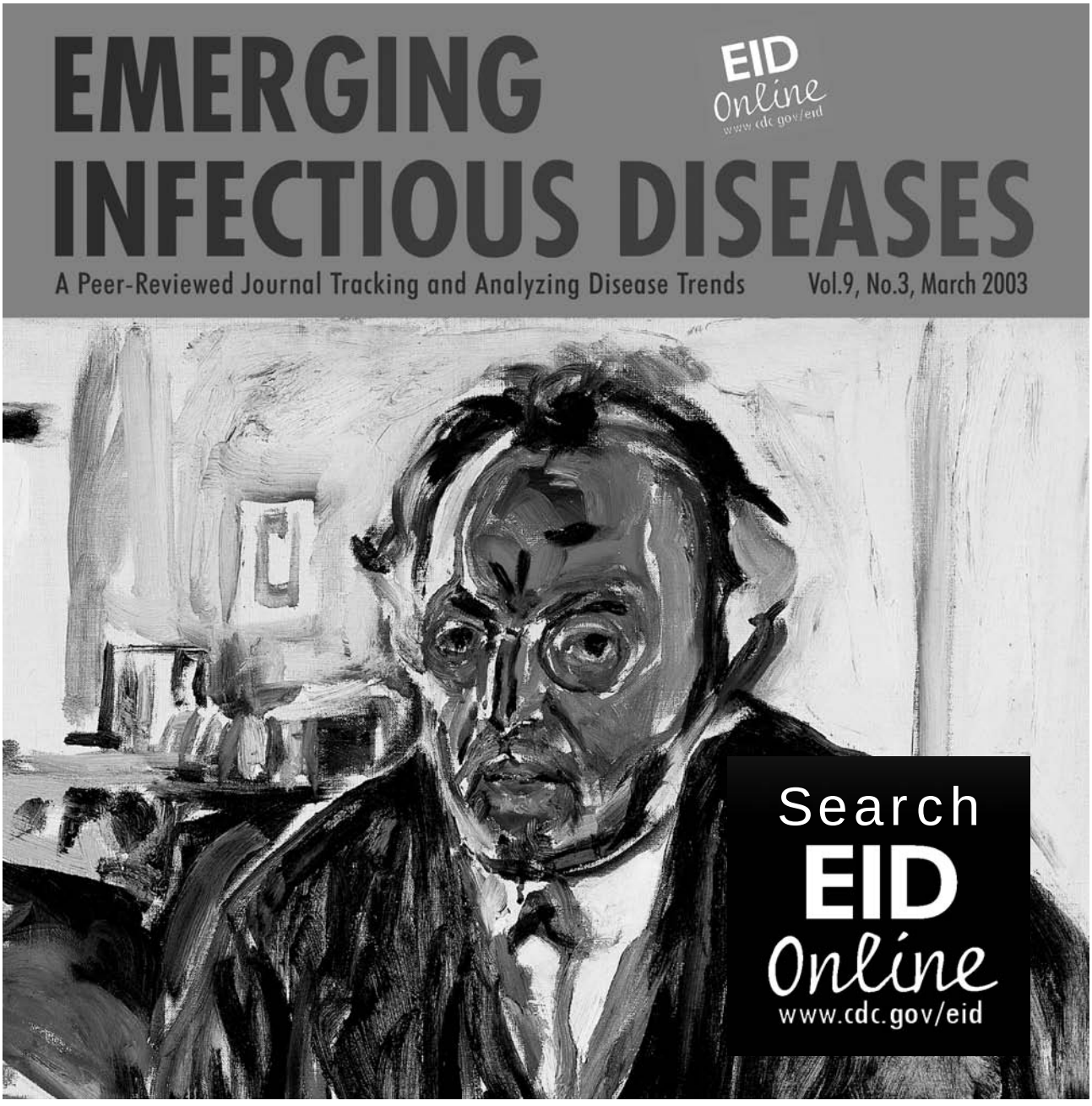

Novel Swine Influenza Virus Subtype H3N1, United States

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Influenza A virus infects various animal species and transmits among different hosts, especially between humans and swine. Swine may serve as a mixing vessel to create new reassortants that could infect humans. Thus, monitoring and characterizing influenza viruses in swine are important in preventing interspecies transmission. We report the emergence and characterization of a novel H3N1 subtype of swine influenza virus (SIV) in the United States. Phylogenetic analysis showed that the H3N1 SIVs may have acquired the hemagglutinin gene from an H3N2 turkey isolate, the neuraminidase gene from a human H1N1 isolate, and the remaining genes from currently circulating SIVs. The H3N1 SIVs were antigenically related to the turkey virus. Lung lesions and nasal shedding occurred in swine infected with the H3N1 SIVs, suggesting the potential to transmit among swine and to humans. Further surveillance will help determine whether this novel subtype will continue to circulate in swine populations.

Influenza A viruses infect many animal species including birds, seals, whales, humans, horses, and swine. Migrating waterfowl are the primordial reservoir. They contain a gene pool of all subtypes of influenza A viruses (*1*), and phylogenetic analysis suggests that transmission of influenza A virus among various species can occur. Interspecies transmission between humans and swine has been documented (*1*). Both human and swine influenza viruses (SIVs) recognize sialyl α2,6-galactose oligosaccharide side chains as the receptor on the host cell surface (*2*,*3*). In addition, swine cells also contain sialyl α2,3-galactose-linkage, the receptor for avian influenza viruses. Experimental and epidemiologic evidence demonstrates that different subtypes of avian influenza viruses can replicate in swine (*4*–*6*). Therefore, swine can be a vessel for reassortment of human and avian influenza viruses (*7*).

The viral structure that binds to the cellular receptor is the receptor-binding site, which is located on the globular part of the hemagglutinin (HA) monomer (*8*). Based on a crystallographic model, the receptor-binding site of the H3 subtype includes conserved residues Tyr98, His193, Glu190, Trp53, and Leu194 (*8*). Two other conserved residues at positions 226 and 228 within the binding pocket determine host range specificity (*3*). Leu226 and Ser228 selectively bind to α2,6 sialosides found on human and swine cells, while Gln226 and Gly228 bind to the α2,3 sialosides found predominantly on avian cells (*3*,*9*,*10*).

Influenza viruses currently circulating in North American swine are subtypes H1N1, H3N2, and H1N2 (*11*). The classical H1N1 viruses have been circulating in the swine population since the Spanish flu pandemic of 1918 (*1*). The first SIV, A/SW/IA/15/30, was isolated in 1930 and is antigenically similar to the 1918 human influenza virus (*12*). From 1930 to 1998, classic H1N1 viruses were the predominantly isolated subtype from US swine. In 1998, a new SIV subtype H3N2 emerged and became established in the North American swine population (*13*,*14*). Genetic analysis showed that it was a triple reassortant virus containing genes from swine, human, and avian influenza viruses. The H3N2 SIV acquired the polymerase basic (PB) protein 1, HA, and neuraminidase (NA) genes from a recent human virus, the PB2 and polymerase acidic (PA) protein genes from avian viruses, and the nucleocapsid protein (NP), matrix (M), and nonstructural (NS) genes from the classic H1N1 swine virus (*13*–*16*). A year later, reassortment between the H3N2 and classic H1N1 SIV resulted in a new subtype H1N2, where the HA of the H3N2 subtype was replaced by the HA from the classic H1N1 virus (*17*). This H1N2 subtype caused respiratory disease in swine and continues to circulate in swine populations (*18*). Recently, wholly avian influenza viruses,

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subtypes H4N6 (*5*), H3N3, and H1N1 (*19*), from water fowl were isolated from diseased swine in Canada; however, no evidence shows that these viruses can be successfully maintained in swine populations. We identified and characterized a new SIV subtype H3N1 that may have arisen from reassortment of an H3N2 turkey isolate, a human H1N1 isolate, and currently circulating swine influenza viruses.

Materials and Methods

Clinical Samples

Two SIV isolates, A/SW/MI/PU243/04 (PU243) and A/SW/IN/PU542/04 (PU542), were obtained from 1 swine herd in southern Michigan and 1 in central Indiana, respectively. A/SW/MI/PU243/04 was isolated from lung tissue of a dead 7-week-old, cross-bred swine that was clinically and histologically diagnosed with viral pneumonia. A/SW/IN/PU542/04 was isolated from the nasal swab of a 14-week-old, cross-bred swine that was coughing, had dyspnea, and was lethargic. Both isolates were submitted for virus isolation to the Animal Disease Diagnostic Laboratory of Purdue University.

Virus Isolation and Subtype Determination

Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium supplemented with 2% fetal bovine serum. The 10% lung homogenate (PU243) and nasal swab preparation (PU542) were applied onto MDCK cells maintained in Eagle's minimum essential medium containing 4 μg/mL trypsin and 0.3% bovine serum albumin (Sigma, St. Louis, MO, USA). Cytopathic effect was observed, and the culture supernatant was tested with an HA assay using turkey erythrocytes. RNA was isolated from the supernatant of virus-infected cells by using Trizol (Invitrogen, Carlsbad, CA, USA), and the viral subtype was determined by using 2 different multiplex SIV subtype-specific reverse transcription–polymerase chain reactions (RT-PCR) (*20*). One set of 4 primers was used to differentiate H1 and H3 of HA, and another set of 4 primers was designed for N1 and N2 discrimination.

DNA Sequencing

Two-step RT-PCR was performed by using universal primers and specific primers for influenza A viruses (*21*). The universal primers 5′-AGC AAA AGC AGG-3′ and 5′-ATG AGA AAC AAG G-3′ were used to amplify NS, M, NA, NP, and HA genes of the 2 isolates. The remaining genes, PA, PB1, and PB2, were amplified by using gene-specific primers. The primer pairs are PA F, 5′- AGC AAA AGC AGG TCA-3′; PA R, 5′-ATG AGA AAC AAG GTA CTT-3′; PB1 F, 5′-AGC AAA AGC AGG CA-3′; PB1 R, 5′-ATG AGA AAC AAG GCA TTT-3′; PB2 F, 5′-AGC AAA AGC AGG TC-3′; PB2 R, 5′-ATG AGA AAC AAG GTC GTT T-3′. RNA was reverse transcribed by using Superscript II (Invitrogen), and the cDNA was amplified by using the expand high fidelity PCR system (Roche, Indianapolis, IN, USA) according to manufacturer's instructions. The PCR products were cloned into pGEMT Easy (Promega, Madison, WI, USA). Purified plasmids containing the viral genes were sequenced by using an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) at the sequencing facility of the National Animal Disease Center, Agricultural Research Services, US Department of Agriculture (Ames, IA, USA). At least 4 cDNA clones of each gene were analyzed.

Phylogenetic Analysis

Individual gene sequences were combined and edited by using Lasergene (DNASTAR, Madison, WI, USA). Megablast (National Center for Biotechnology Information, Bethesda, MD, USA) searches were performed to identify sequences with the best match to each individual gene of the 2 H3N1 viruses. Multiple alignments of DNA sequences were conducted on the complete NA gene and the HA1 region of the HA gene by using ClustalW (DNASTAR). Maximum parsimony phylogenetic trees were created by using MEGA3 (The Biodesign Institute, Tempe, AZ, USA) (*22*). The HA tree was rooted by an unrelated H4 duck influenza virus, A/duck/Alberta/28/76. An avian N2, A/chicken/CA/6643/01, represented the outgroup of the NA tree. Each tree is a consensus of 1,000 bootstrap replicates.

Hemagglutination Inhibition (HI) Assay

HI assays were performed to determine the antigenic relationship between the 2 H3N1 viruses, the H3N2 turkey isolates (*23*), and H3N2 SIVs. The H3N2 SIVs tested in the HI assay included viruses representing 3 H3N2 clusters: cluster I, TX98 (A/SW/TX/4199-2/98); cluster II, CO99 (A/SW/CO/23619/99); and cluster III, WI99 (A/SW/WI/14094/99) and IL99 (A/SW/IL/ 21587/99). Swine hyperimmune sera against various H3N2 SIVs (*24*) and a ferret serum raised against an H3N2 turkey isolate were adsorbed with kaolin powder to eliminate nonspecific inhibitors. The 2 H3N1 and 4 H3N2 SIVs were tested with respective sera in a standard HI assay (*25*).

Experimental Animal Infection

The 2 H3N1 viruses were inoculated into 10-week-old cross-bred swine in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. The protocol for infection is described elsewhere (*24*). Briefly, 2 groups of swine (n = 4 or 5) were infected intratracheally with 2×10^5 PFU/swine of either

A/SW/MI/PU243/04 or A/SW/IN/PU542/04 inoculum (total of 1 mL) prepared in embryonated eggs. Four swine were mock infected with medium only and served as controls. Five days after infection, swine were euthanized, lung lesions were scored (*24*), and bronchoalveolar lavage fluid (BALF) was collected. Sera and nasal swabs were collected the day of and 5 days after infection.

Virus load in BALF, serum samples, and nasal swabs were determined in a 96-well format (*24*). Each sample was serially diluted 10-fold and injected into a monolayer of MDCK cells. The infected cells were fixed with methanol 48 hours after infection, and an indirect immunofluorescence assay was conducted by using anti-SIV swine serum (primary antibody) and a secondary fluorescein isothiocyanate–conjugated anti-swine antiserum (Sigma). Wells were determined as either positive or negative without counting individual foci. The virus titers were determined as 50% tissue culture infective dose ($TCID_{50}$) per milliliter.

Detecting Swine Respiratory Pathogens

The presence of porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* in BALF was determined by using either RT-PCR or PCR assays, respectively. For PRRSV, total RNA was isolated from BALF from each swine by using the QIAamp Viral RNA mini kit (Qiagen, Valencia, CA, USA). One microgram of the extracted RNA and a primer pair specific for open reading frame 5 of PRRSV were included in a single-tube RT-PCR as described previously (*26*). To find *M. hyopneumoniae*, DNA was extracted from BALF by using the QIAamp DNA mini kit according to the manufacturer's recommendations (Qiagen). A forward primer specific for *M. hyopneumoniae* and a common reverse primer for the 16S rRNA gene were used in the PCR as previously described (*27*). A laboratory-grown *M. hyopneumoniae* DNA sample was used as a positive control. Amplified products were detected by electrophoresis on ethidium bromide–stained agarose gel.

Results

Virus Isolation and Subtype Determination

MDCK cells injected with a lung homogenate from swine PU243 or with the nasal swab of swine PU542 produced cytopathic effect approximately 2–3 days after infection. The supernatant agglutinated turkey erythrocytes in HA tests. Total RNA of each isolate was prepared from the supernatant of PU243- or PU542-infected MDCK cells and used as templates for the multiplex RT-PCR. Results of the multiplex RT-PCR assay specific for HA showed that both isolates were of the H3 subtype, since no H1-specific band was present. The multiplex RT-PCR specific for NA showed that both isolates were of N1 and not N2 subtype. The 2 H3N1 SIV isolates were designated A/SW/MI/PU243/04 or A/SW/IN/PU542/04. Subsequently, the RNA from the culture supernatants was used for amplification and cloning.

Experimental Animal Infection

Four or five 10-week-old swine, negative for SIV-specific antibodies, were infected with the PU243 and PU542 H3N1 isolates. No respiratory difficulties were reported during 5 days of observation before the animals were euthanized. At necropsy, macroscopic lesions characterized by marked plum-colored, consolidated areas on lung lobes were observed. The PU243-infected swine had an average lung lesion score of ≈8%. The PU542 infected group had a milder lung lesion score of ≈3%. Control swine had no obvious lung lesions.

To determine lung replication and nasal shedding of the H3N1 viruses in swine, virus titers in the sera, nasal swabs, and BALF were evaluated. All samples from all swine in the control group as well as all samples obtained before infection were virus negative. Titers of viruses from nasal swabs and BALF 5 days after infection are shown in Table 1. Viral loads in BALF at 5 days after infection ranged from $10^{6.3}$ to $10^{7.6}$ $TCID_{50}$/mL (mean 10^{7}) and were substantially greater ($p<0.05$) than those in nasal swabs 5 days after infection (titer range 10^{3}–$10^{5.8}$ $TCID_{50}$/mL, mean $10^{4.7}$ $TCID_{50}$/mL). Sera from infected swine collected 5 days after infection were virus negative.

Other respiratory pathogens of swine that might produce lung lesions similar to SIV were not found in BALF of infected swine. The result of a PCR specific for the 16S RNA of *M. hyopneumoniae* showed that BALF from all swine were negative. Similarly, BALF did not contain PRRSV nucleic acids. These results eliminated the possibility that swine might have been infected with *M. hyopneumoniae* or PRRSV.

Sequence Analysis

Comparison of individual gene sequences of the 2 H3N1 SIVs showed that the identities ranged from 92.3% to 99.3% at the nucleotide level. The M gene is the most conserved, while the HA and NA genes are more variable with identities of 96.5% and 92.3% at the nucleotide level and 95.6% and 92.3% at the amino acid level, respectively. The similarity among the remaining 5 genes of the 2 isolates is >98%. Table 2 shows results obtained from Megablast analyses, which searched for sequences in the GenBank with the best match to each individual gene of both H3N1 SIVs. The HA of both H3N1s has the highest similarity with the HA of an H3N2 virus isolated from a turkey (A/TK/NC/12344/03). The NA sequence of both H3N1s is closely related to the NA of a human H1N1 isolate (A/WI/10/98) (*28*). The remaining 5 genes of both

Table 1. Virus titers in nasal swabs and BALF from experimentally infected pigs 5 days after infection*

Inoculum/	A/SW/MI/PU243/04 ($TCID_{50}$/mL)					A/SW/MI/PU542/04 ($TCID_{50}$/mL)			
pig no.	108	109	111	114	127	3	110	112	113
Nasal swab	$10^{4.3}$	$10^{4.7}$	10^{5}	10^{3}	$10^{5.8}$	$10^{5.8}$	$10^{5.6}$	$10^{4.5}$	$10^{4.1}$
BALF	$10^{6.6}$	$\geq 10^{7.3}$	10^{7}	$\geq 10^{7.6}$	$10^{6.3}$	$\geq 10^{7.5}$	$10^{6.5}$	$10^{7.6}$	$10^{6.9}$

*BALF, bronchoalveolar lavage fluid; $50\%TCID_{50}$, 50% tissue culture infective dose.

isolates are closely related to respective genes found in currently circulating H3N2 and H1N2 SIVs (Table 2). The M gene of PU243 isolate is most similar to a turkey isolate (A/TK/NC/12344/03) while the M gene of PU542 is similar to an H1N2 SIV (A/SW/IN/14810-S/01).

Phylogenetic Analyses

Maximum parsimony analysis of the HA1 region of the H3 subtype of recent North American SIVs separates these subtypes into 3 clusters as previously reported (Figure 1A) (*16*). Both H3N1 SIVs are closely related to 2 H3N2 turkey isolates, A/TK/NC/16108/03 and A/TK/MN/764/03, within cluster III. Branch length between the H3N1 viruses and the turkey H3N2 viruses is shorter than between the H3N1 SIVs and the swine H3N2 isolates.

Phylogenetic analysis of the N1 subtype of NA separates the sequences into 3 groups: swine, human, and avian. Both human and swine N1s share a common ancestor; however, they are placed in different clusters (Figure 1B). The N1s of the 1930 and 1973 SIVs were placed near the root of the human cluster. The human influenza isolates within the swine group were obtained from humans infected with swine viruses. The NAs of both H3N1 SIVs are placed into the swine cluster and are most closely related to an H1N1 human virus, A/WI/10/98. The A/SW/IN/PU243/04 and the 1998 H1N1 human isolate were placed in similar root at a 99% level.

Antigenic Relationship of Swine and Turkey H3 Subtype Viruses

Cross-reactivity between the H3N1 SIVs, H3N2 turkey isolates, and H3N2 SIVs representing 3 genetic clusters (*16*) were tested in HI assays. The results showed that neither H3N1 isolate reacted with antibodies raised against H3N2 swine viruses representing cluster II and III at a 1:10 dilution, the lowest dilution tested. They reacted poorly with a serum raised against the cluster I TX98 SIV, with an HI titer of 20. PU243 reacted with a ferret serum raised against the H3N2 turkey isolate with a low HI titer of 40. PU542 reacted weakly with the same ferret serum with an HI titer of 20. Antisera from swine infected with each H3N1 virus showed weak reactivity to a H3N2 cluster I SIV (TX98) with an HI titer of 10 and cluster III SIVs (WI99 and IL99) with HI titers of 20 and moderate reactivity to a cluster II SIV (CO99) with HI titers of 80 for PU243 and 40 for PU542.

Receptor-binding Site

Critical amino acid positions within the receptor-binding site of the H3 subtype of swine and turkey viruses are shown in Figure 2. Most of the residues are highly conserved, especially those associated with the sialoside receptor-binding region, Tyr98, Trp153, His183, Glu190, and Leu194 (*29*,*30*), and the residues (amino acids 226 and 228) responsible for host range specificity (*3*,*10*). All isolates have Tyr98, Trp153 (with the exception of H3N1 PU542), His183, Asp190 or Glu190, Leu194 (with the exception of H3N2 TX98), and Ser228. Residue 226 of the H3 subtype of SIVs and the H3N2 turkey viruses is either Ile or Val instead of Leu (Figure 2).

Discussion

Although influenza viruses show host-range–specific

Table 2. Results of Megablast nucleotide analyses of influenza A viruses with the best match of each gene with the H3N1 swine influenza viruses*

	A/SW/MI/PU243/04			A/SW/IN/PU542/04		
Gene	Virus	Subtype	% identity	Virus	Subtype	% identity
PB2	A/SW/IL/10084/01	H1N2	98.20	A/SW/IL/10084/01	H1N2	98.20
PB1	A/SW/IA/930/01	H1N2	98.37	A/SW/IA/930/01	H1N2	97.93
PA	A/SW/IA/569/99	H3N2	97.21	A/SW/IA/569/99	H3N2	97.02
HA	A/TK/NC/12344/03	H3N2	96.91	A/TK/NC/12344/03	H3N2	97.42
NP	A/SW/OH/891/01	H1N2	98.96	A/SW/OH/891/01	H1N2	98.46
NA	A/WI/10/98	H1N1	95.54	A/WI/10/98	H1N1	93.78
M	A/TK/NC/12344/03	H3N2	98.78	A/SW/IN/14810-S/01	H1N2	99.48
NS	A/SW/IN/14810-S/01	H1N2	99.24	A/SW/IN/14810-S/01	H1N2	99.05

*Accession numbers: A/SW/MI/PU243/04 PB2 (DQ150422), PB1 (DQ150423), PA (DQ150424), HA (DQ150425), NP (DQ150426), NA (DQ150427), M (DQ150428), and NS (DQ150429); A/SW/IN/PU542/04 PB2 (DQ150430), PB1 (DQ150431), PA (DQ150432), HA (DQ150433), NP (DQ150434), NA (DQ150435), M (DQ150436), and NS (DQ150437); A/SW/IL/10084/01 PB2 (AF455738); A/SW/IA/930/01 PB1(AF455727); A/SW/IA/569/99 PA (AF251425); A/TK/NC/12344/03 HA (AY779253) and M (AY779257); A/SW/OH/891/01 NP (AF455699); A/WI/10/98 NA (AF342820); A/SW/IN/14810-S/01 M (AY060071) and NS (AY060136).

characteristics, interspecies transmission of influenza viruses has been well documented (*1*). Infection of turkeys with swine influenza viruses seems to be common, and influenza viruses isolated from turkeys indicated that 73% of turkey influenza viruses contained genes of swine origin (*31*). Influenza viruses antigenically similar to the classic H1N1 swine virus were found to infect and produce diseases in different turkey herds (*32–34*). Recently, an influenza virus containing 8 genes closely related to those of A/SW/IN/9K035/99 H1N2 caused an outbreak in a turkey flock from Missouri (*35*). Thus far, transmission of turkey viruses to swine populations has not been reported.

SIV subtype H3N1 viruses were previously isolated in Taiwanese swine; these viruses most likely acquired the HA from a human H3N2 isolate and the NA from an H1N1 SIV circulating in Taiwan (*36*). The novel H3N1 SIVs reported here contain HA genes highly similar to those of recently reported H3N2 turkey isolates. These H3N2 turkey isolates, A/TK/NC/16108/03 and A/TK/MN/764/03, were most likely swine viruses, which infected and caused disease in turkeys (*23*). Phylogenetic analysis of the HA1 region of the HA gene placed the H3N1 SIVs at a similar root to the turkey isolates. Additionally, branch lengths of the H3N1 SIVs and the turkey isolates are shorter than those between the H3N1 viruses and the swine H3N2 viruses. This finding suggests that the H3N1 SIVs may have acquired their HA from a virus similar to the H3N2 turkey isolate; this finding could indicate interspecies transmission from turkeys to swine. Subsequently, the swine H3N1s have diverged separately from the turkey H3N2s.

Maximum parsimony analysis of the NA gene separates human, avian, and swine clusters as previously reported (*37*). The NA of the H3N1 SIVs is placed into the swine cluster. A/SW/MI/PU243/04 shares a similar root with the human H1N1 isolate, WI/10/98, at the 99% level. This finding strongly suggests that both viruses have a common ancestor; however, the H3N1 swine virus may have evolved from the WI/10/98 H1N1 or similar human isolates. Although the 2 H3N1 SIVs were isolated from 2 separate herds, they may have evolved from a similar ancestor. Both HA and NA phylogenetic analyses placed the 2 isolates into different branches at 61% and 52% bootstraps, respectively. Both may have originated from a similar reassortant event and continued diverging from each other. Analysis of the deduced amino acid sequence (Figure 2) also supports this assumption.

Residues mainly responsible for sialyl α2,6-galactose specificity are Leu226 and Ser228 (*3*,*10*). Leu226 is not in contact with the sialoside but changes in this position alter the conformation of the binding pocket (*30*). The space-filling model of the H3 HA complex with a receptor analog showed that Leu226 is in close proximity to the Van

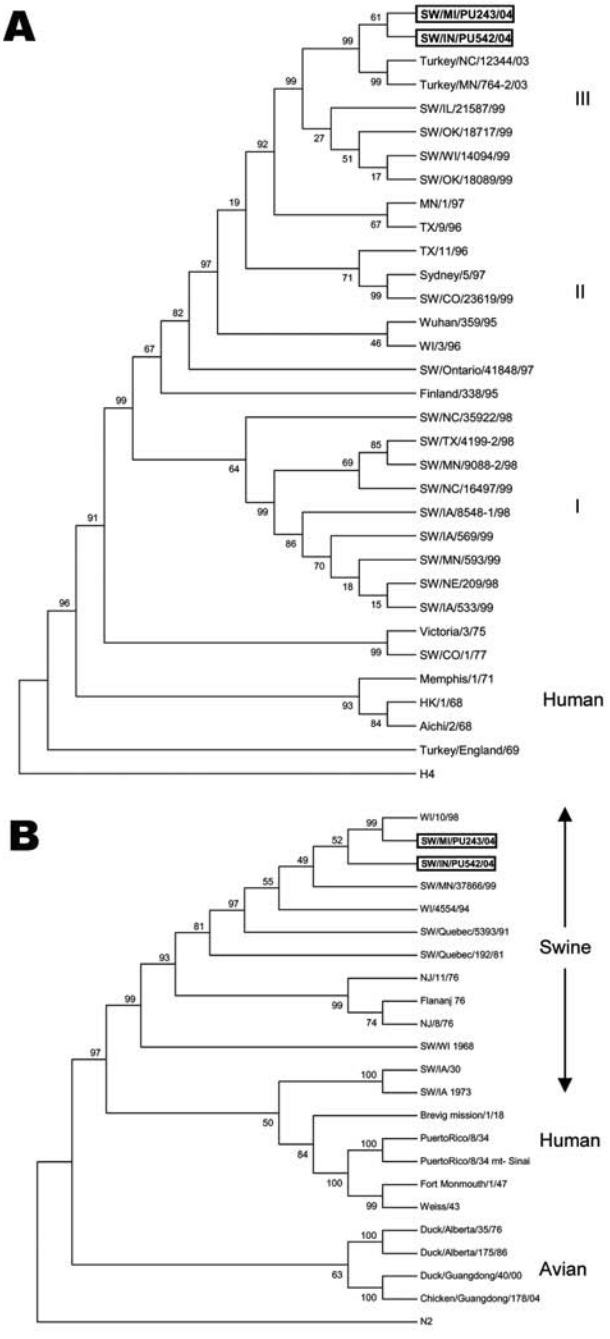

Figure 1. Genetic relationships of the hemagglutinin (HA) 1 region of HA gene and neuraminidase (NA) gene of the H3N1 swine influenza viruses (SIVs) with other influenza viruses. The tree was created by maximum parsimony method and bootstrapped with 1,000 replicates. The bootstrap numbers are given for each node. A) Phylogenetic trees demonstrating genetic relationship of the closely related H3N2 turkey isolates, recent H3N2 SIVs, and human H3N2s. The tree was created from the HA1 region of HA and rooted to an unrelated sequence, the H4 HA of A/duck/Alberta/28/76. B) Phylogenetic analysis of the N1 subtype of NA genes of human, swine, and avian influenza viruses. N2 of A/Chicken/CA/6643/01 was used as an outgroup sequence.

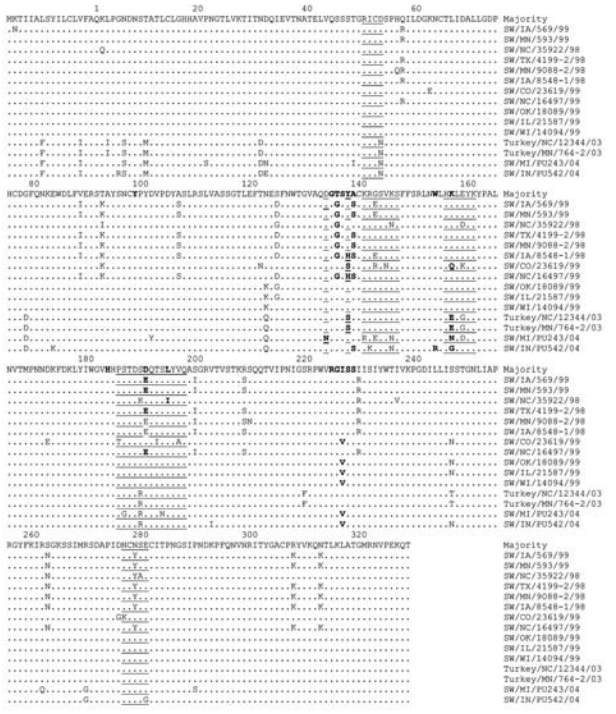

Figure 2. Alignment of deduced amino acid sequences within the hemagglutinin (HA) 1 region of HA genes of H3N2 swine influenza viruses (SIVs), H3N2 turkey isolates, and H3N1 SIVs. The amino acid sequence represents the consensus sequence, and the amino acid at position 1 is the first amino acid following the signal peptide (*37*). Dots represent amino acids similar to the consensus. Note that according to H3 structure (*37*), the residues representing the antigenic sites are underlined and the receptor binding sites are in **boldface**. The alignment shows that PU243 and PU542 may have emerged from the H3N2 turkey isolates. The residues within the receptor-binding site are relatively conserved.(For a larger version of this figure, see online article, available at http://www.cdc.gov/ncidod/EID/vol12no05/05-1060-G2.htm)

der Waal space of C6 of the galactose (*29*). The H3N1 and H3N2 SIV sequences, including the H3N2 turkey viruses, contain all Ser228 but Ile226 or Val226 instead of Leu226 in their HA1 molecules. H3 subtypes of human influenza viruses isolated from Japan and China during 1994 and 1995 also contain Ile226 instead of Leu226 (*38*). Leu, Ile, and Val are similar neutral nonpolar amino acids; substitution between them most likely maintains hydrophobic interactions and proper conformation of the binding pocket. In contrast, Gln226 and Gly228 are normally found in the HA1 molecule of avian viruses (*10*). Gln is classified as a hydrophilic amino acid, and its amino acid structure is different from Leu, Ile, or Val. Ser and Gly are classified into different groups of amino acids; they possess different charges and structure. The H3 turkey HA still maintains Ile226 and Ser228 similar to that of swine viruses, indicating that they maintained their ability to infect swine and possibly humans, despite replicating in an avian host. Whether influenza virus receptors in turkeys are different from those in other avian species is not known.

Why it took ≈6 years for H3N1 SIVs to emerge in US swine where H3N2 and H1N1 viruses have been cocirculating since 1998 is not known. Reassortant H1N2 and H1N1 SIVs were isolated shortly after the 1998 introduction of the H3N2 viruses into US swine. A certain constellation of the HA and NA surface molecules was necessary to create a successful H3N1 reassortant, since optimal balance between NA activity and HA affinity to the sialoside receptor is crucial for effective influenza virus infections (*39–41*).

Our study showed that the H3N1 SIVs can replicate in the respiratory tract of swine and are shed in nasal secretions. In this study, investigations on virus transmissibility in which contact animals are housed together with infected animals were not performed. Therefore, whether these 2 H3N1 SIVs will be transmitted efficiently in the field situation requires further experimental and epidemiologic studies. However, our results underline the scenario in which swine can be a mixing vessel for human, swine, and avian influenza viruses to create new reassortants that may be dangerous to human health. Turkeys are more susceptible to influenza viruses from waterfowl than are other domestic poultry (*42*), and a high degree of genetic reassortment most likely occurs in domestic turkeys (*31*). This finding may indicate that influenza A viruses could sequentially acquire new genes during transmission from waterfowl via turkey to swine and humans.

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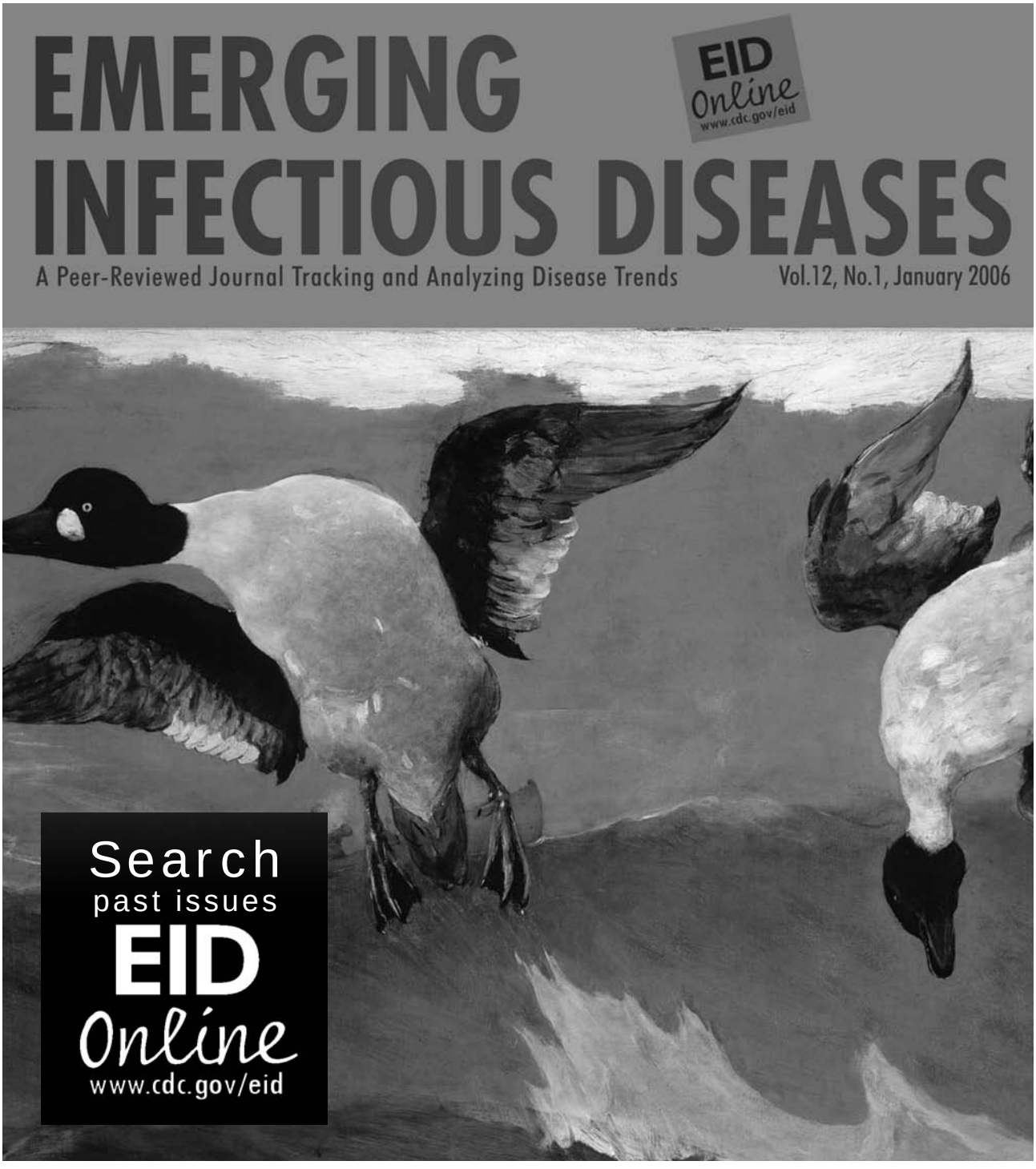

The Trojan Chicken Study, Minnesota

Sandra R. Olson*† and Gregory C. Gray*

We conducted a study in the summer of 2004 at county fairs in the Midwest to investigate the role poultry exhibits have in spreading avian pathogens to humans. A nearly invisible powder (pathogen surrogate) that fluoresces under UV light was surreptitiously sprinkled each day on 1 show bird at each of 2 fairs. A UV light box was used to daily examine the hands of 94 poultry-exhibit participants (blinded regarding UV box results) for up to 4 days during the poultry shows. Enrollment and end-of-study questionnaires collected data on pathogen risk factors. Eight (8.5%) of 94 participants had evidence of fluorescent powder contamination (95% confidence interval 2.76%–14.26%). This contamination and infrequent handwashing practices suggest that county fairs are a possible venue for animal-to-human pathogen transmission.

Recently, the Centers for Disease Control and Prevention (CDC) declared avian influenza to be the world's number-1 health threat (*1*); in particular, the wide and rapid spread of the H5N1 strain has heightened concerns. All H5N1 cases to date have been associated with direct contact with poultry, but recently, human-to-human transmission has been purported in Thailand (*2*). Previously healthy children and young adults seem to be especially susceptible to this illness (*3*). As of February 27, 2006, a total of 173 confirmed human cases of avian influenza A (H5N1) and 93 deaths have been reported to the World Health Organization, for a case-fatality rate of 53.8% (*4*).

Close contact with live poultry has been implicated in recent outbreaks of avian influenza in humans in Southeast Asia and elsewhere (*2*,*5*–*8*). In the 1997 Hong Kong outbreak, live bird markets were implicated as the source of exposure to the virus (*8*). In the United States, live bird markets are a known reservoir for avian influenza (*9*–*11*), but thus far they have not been associated with human avian influenza infection. Live bird markets involve a mixing of birds from diverse areas, crowded conditions for humans and livestock, mixing of different species of animal, and often a lack of proper sanitation, thus providing opportunity for outbreaks of disease. Transport of animals to market is a source of stress than can induce increased shedding of infectious agents. Stressed birds are also more susceptible to infections (*12*).

While live bird markets are uncommon in the Midwest, animal exhibits such as those at county fairs are quite common. Such exhibits are similar to live bird markets in that they involve transport and mixing of animals from different locations, crowded conditions, and a general lack of sanitation. Approximately 125 million people visit agricultural fairs every year in the United States (*13*). Fairs usually involve close proximity of food vendors to animal exhibits. Many animal exhibits encourage or allow visitors to touch animals. Small children are frequent visitors to county fairs and animal exhibits, and children also engage in behavior such as nail biting that may make them more likely to ingest infectious agents. Live animal exhibits such as petting zoos and open farms, which are in many ways similar to county fairs, have also been implicated in outbreaks of *Escherichia coli* O157:H7 and other bacterial diseases (*13*,*14*).

Proper handwashing is recommended to protect persons from infection (*15*). However, animal exhibits often lack adequate handwashing facilities, and many persons may be unaware of the risk such exhibits pose. Direct contact with animals, indirect contact with contaminated objects, or inhalation of aerosolized virus could contribute to transmission of pathogens in such settings.

Because little is known about the possible spread of pathogens at county fairs, and because most cases of avian influenza have resulted from close contact with poultry, a study was undertaken to model interspecies transmission of pathogens at county fair poultry shows. The specific aims of this study were to determine the proportion of

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human poultry show participants who demonstrate hand contamination by a surrogate marker for an avian pathogen and to determine possible risk factors associated with such contamination.

Materials and Methods

A feasibility study was conducted at a county fair in Iowa (county A) to evaluate study methods. After the feasibility study, human poultry fair participants were enrolled at a larger county fair in Minnesota (county B). Both fairs were held within small cities with populations of ≈100,000.

At county fairs, poultry judging often takes place in show areas that are open to the public. Birds are usually placed in cages that are stacked one upon another and set upon tables (Figure). Because poultry classes are judged separately and competitors may show their birds in several poultry classes, birds are frequently moved in and out of their cages for grooming and competition. During the competition, birds are moved to competition cages that have previously housed birds from other competition classes. Judges typically handle each bird individually; they take the bird from the exhibitor, examine it, and then hand it back to the exhibitor (Figure). Handwashing is not generally performed as the judge moves from bird to bird, nor is handwashing common before or after exhibitors handle their birds. After competition, birds often remain on exhibit for several days, and they may be touched by the general public.

This study was reviewed and approved by the University of Iowa's Institutional Review Board and Animal Use and Care Committee. The investigators participated in online human and animal subjects training. Informed consent was sought from participants before they were enrolled.

Anyone ≥7 years of age present in the poultry exhibit area at any time during the period when poultry were on active exhibit was eligible to enroll in the study. Recruitment focused on members of 4-H clubs and open-class exhibitors, their families, and 4-H club staff, but also included other visitors. Enrollment occurred continuously over a 4-day period (Monday through Thursday) while poultry were exhibited at the fairs. A special sign and an information table were used to promote the study. Study participants were recruited for enrollment as they walked through the poultry exhibit area. After providing informed consent, study participants were asked to complete a 1-page questionnaire that gathered demographic and poultry exposure data. Participants were also asked to complete a 1-page end-of-study questionnaire after they completed their experience at the poultry exhibit (day 4). This instrument gathered data on handwashing and types of animals handled at the fair.

GloGerm (GloGerm Company, Moab, UT, USA), a benign, synthetic, organic colorant A-594-5 that fluoresces under a black light, was used as a surrogate marker for an avian pathogen. This powder (also found in liquid or gel form) is commonly used in handwashing training in hospitals and businesses (*16*). Each day, the white powder was surreptitiously applied to the same single chicken at the fair to imitate a single source of pathogen. White broiler chickens were chosen as the exposure birds since the powder was not detectable on their feathers. Each "Trojan chicken" was otherwise treated the same as the other chickens in the poultry shows. While county fair authorities gave permission for the study, neither the judge nor the study participants were aware of neither the surrogate exposure nor which of the chickens were of particular hygienic concern. Instead several participants remarked that they thought the UV light box (see below) in which

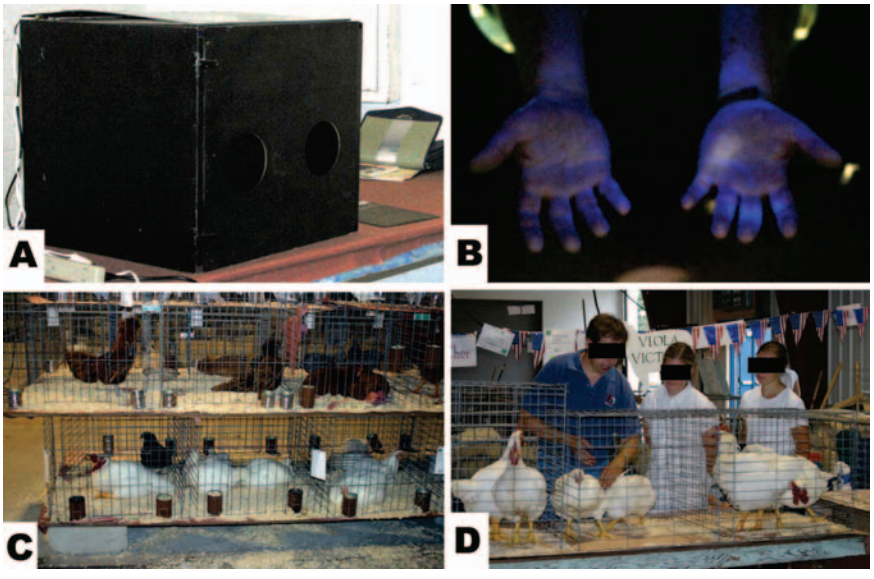

Figure. A) UV light box for screening hands for evidence of contamination with fluorescent dye; B) example of fluorescence on contaminated hands; C) stacked poultry in cages at a county fair; D) poultry judge moved from cage to cage handling each bird and passing bird to exhibitor.

photographs were taken could somehow detect generic bacterial contamination on the hands.

At county A, chicken powdering was conducted early in the mornings of the 3 days of competition, when competitors were not at the poultry exhibit. At county B, the same strategy was followed but the Trojan chicken was also surreptitiously powdered again in the early afternoon for 3 days of the show. During the powdering, approximately one-third cup powder was liberally sprinkled onto the underside of the chicken to imitate fecal shedding of pathogen. The chicken was then returned to its cage. The Trojan chickens each shared their cage with another, very similar, white broiler chicken, since these birds are normally shown in matched pairs.

To evaluate potential avian influenza transmission, a 2 × 2 × 2-foot wooden box was constructed from plywood. Three black 1-foot × 18-inch fluorescent lights (15 watts) and 1 white 1-foot × 18-inch fluorescent light (15 watts) were mounted under the lid of this isolation box. Study participants inserted their hands through hand holes in 1 side, and they were blinded as to the result of the fluorescence examination of their hands. From an opening in the box on the opposite site, digital photographs of the ventral and dorsal images of the hands were taken with a digital camera (Figure). A log was kept to match the sequentially captured photograph numbers with the participants' names (data were later de-identified). Beginning on day 1 of each poultry show, daily photographs were taken of study participants' hands under the black lights (Figure). Photographs continued to be taken through the afternoon of day 4 (last day of the shows).

Statistical analysis was performed with SAS version 8.0 (Cary, NC, USA). Chi-squared analysis and Fisher exact test were used to compare categorical variables with powder contamination. We used *t* tests to compare continuous variables. Logistic regression modeling was attempted, but the models did not converge. Odds ratios and confidence intervals were calculated by using EpiInfo (CDC, Atlanta, GA, USA) (Table 1).

Results

Ninety-four persons participated in the study by having their hands photographed. Among these were 30 poultry exhibitors (Table 2). Of the study participants, 82 (87.2%) completed the enrollment questionnaire, and 44 (46.8%) completed the end-of-study questionnaire. Of all participants in county B, 29 (30.9%) were male.

The mean age of those who completed the enrollment questionnaire was 33 (range 7–79) years. Eighteen participants were poultry exhibitors, who showed 1–10 birds each (mean 3.4). Fifty-five participants (67.1%) of 82 were residents of farms.

Eight participants exhibited hand contamination (Table 1). Of these, all 8 completed the enrollment questionnaire, and 7 completed the end-of-study questionnaire.

Participant gender and hand contamination were not associated. Of participants whose hands were contaminated, 3 were male and 5 were female. None of the persons whose hands were contaminated were exhibitors: 3 were family members of exhibitors, 3 were visitors, and 1 was in the "other" category.

In the age group of 7 to 12 years, 1 (7.7%) participant had hand contamination (Table 1). None of the participants in the 13- to 21-year age group showed hand contamination. Four participants (10.8%) in the 22- to 50-year age group had contaminated hands, and 2 participants (14.3%) who were ≥51 years showed hand contamination. Contamination rates did not differ by age group.

Discussion

Our study demonstrated that pathogen transmission is possible through poultry handling at county fairs. A contact transmission proportion of 8.5% (8 persons of the 94 participants had contaminated hands) is high, when one considers the insensitivity of the measure (gross fluorescence) and the number of persons possibly exposed at a

Table 1. Hand contamination by variables sex, age, and roles*

Variable	Not contaminated, n = 86 (%)	Contaminated, n = 8 (%)	OR (95% CI)
Sex			
Female	60 (92.3)	5 (7.7)	Referent
Male	26 (89.7)	3 (10.3)	1.4 (0.2–7.7)
Age group, y			
7–12	12 (92.3)	1 (7.7)	0.3 (0.0–3.3)
13–21	18 (100)	0 (0)	Referent
22–50	33 (89.2)	4 (10.8)	0.4 (0.1–2.3)
51–79	12 (85.7)	2 (14.3)	0.6 (0.1–4.5)
Role			
Exhibitor	18 (100)	0 (0)	Referent
Family member of exhibitor	14 (82.4)	3 (17.6)	0.8 (0.1–4.8)
Visitor	35 (92.1)	3 (7.9)	0.3 (0–1.8)
Other	8 (88.9)	1 (11.1)	0.5 (0–5.2)

*OR, odds ratio; CI, confidence interval. All OR were calculated with exact CI by EpiInfo version 3.3.2 (CDC, Atlanta, GA, USA). The value 0.5 was inserted into cells with values of zero.

Table 2. Characteristics of participants (N = 94)

Characteristic	n (%)
Completed questionnaire 1	82 (87.2)
Completed questionnaire 2	44 (46.8)
Sex	
Male	29 (30.9)
Female	65 (69.1)
Farm resident	63 (67.0)
Role	
Exhibitor	18 (22.0)
Family member of exhibitor	17 (20.7)
Visitor	38 (46.3)
Other	9 (11.0)
Age, y	
Mean 33	
SD 17.9	
Range 7–79	

county fairs. Both male and female participants were affected, as well as most age and role groups.

This study had some unique characteristics. Digital photography of a fluorescent powder on hands was a successful surrogate for contamination. However, this rather gross measure was likely insensitive when one considers how few bacterial or viral particles are needed to cause certain zoonotic diseases. The black light box was also successful in blinding participants to their contamination status, since they were unable to see inside the box, and few seemed to grasp the experimental nature of the study.

Some of our study findings were unanticipated. We expected contamination proportions to vary by age, gender, and role because we expected these factors to affect the amount of contact with birds and handwashing behavior. However the rates did not vary by these variables. This finding could be due to the study's limited power to detect such differences. If the differences between those exposed and those unexposed were statistically significant (e.g., also occurring in a similar study with a larger sample size), they might be consistent with studies that suggest that animal handlers (exhibitors) practice better hygiene compared to nonhandlers in the same environment. Alternatively, animal handlers may engage in other behavior that affects their contamination status, such as handling enough animals that the surrogate powder wears away more quickly than it would for someone who does not handle animals.

This theoretical model had limitations. Hand contamination with the fluorescent powder was considered a surrogate for pathogen transmission in this study; however, hand contamination of a pathogen does not necessarily lead to transmission. Transmission is dependent upon the amount of inoculated pathogen (dose), the ability of the pathogen to cause disease (virulence), and the ability of the host to defend against infection (host susceptibility) (*17*). These variables are complex and difficult to measure in settings such as a county fair. Additionally, such variables often vary by pathogen and host; hence, we measured only surrogate markers for exposure because such exposure is a requirement for disease to occur.

GloGerm powder contamination may or may not be reflective of true pathogen transmission. The product is useful in handwashing training because it is generally not visible to the naked eye and persons are usually unaware that they have become contaminated. In our study, GloGerm was additionally useful because study participants were also unaware that a chicken was contaminated. Proper handwashing removes the powder, as it would pathogens. However, the amount of time the powder remains on a person's hands without handwashing varies and may be different from the amount of time that a pathogen would be viable on hands. In addition, dusting the chicken with powder is an attempt to model pathogen shedding, but this practice may not truly reflect the amount of pathogens an infected bird would shed. The undersides of the birds were dusted to model fecal shedding and dispersal of pathogens. However, the amount of powder used may be higher or lower than true pathogen shedding.

The study design was further limited in that we did not account for time after exposure when photographs were taken. Since participants could drop by any time of the day, the time after exposure and duration of exposure likely varied between participants. In both the feasibility and pilot studies, the return rate was low, and tracking down participants was difficult. If similar studies are conducted in the future, a reward system might be used to increase compliance.

Petting zoos and agricultural fairs are common in the Midwest and attract many thousands of people. While concern about viral and bacterial zoonotic disease transmission in these settings is growing, they are not usually thought of as a public health concern. The observations from this modest study, even with the limitations described above, suggest that live poultry exhibits may pose a disease transmission risk. Of particular concern is the relatively high proportion of powder transmission to poultry show visitors, who have casual and limited exposure to poultry. Larger future studies of similar design might help identify specific risk factors for zoonotic disease transmission and appropriate interventions for such settings. As a minimum contribution, these study data suggest that hygienic educational programs and disease prevention programs are warranted in poultry exhibits.

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Ms Olson conducted this research in partial fulfillment of her master's degree in population health at the University of Wisconsin-Madison. She is now working in the Episense Program, Department of Ophthalmology, at the University of Wisconsin-Madison. She has participated in poultry exhibits for >10 years.

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Aedes aegypti Larval Indices and Risk for Dengue Epidemics

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We assessed in a case-control study the test-validity of *Aedes* larval indices for the 2000 Havana outbreak. "Cases" were blocks where a dengue fever patient lived during the outbreak. "Controls" were randomly sampled blocks. Before, during, and after the epidemic, we calculated Breteau index (BI) and house index at the area, neighborhood, and block level. We constructed receiver operating characteristic (ROC) curves to determine their performance as predictors of dengue transmission. We observed a pronounced effect of the level of measurement. The BI_{max} (maximum block BI in a radius of 100 m) at 2-month intervals had an area under the ROC curve of 71%. At a cutoff of 4.0, it significantly (odds ratio 6.00, $p<0.05$) predicted transmission with 78% sensitivity and 63% specificity. Analysis of BI at the local level, with human-defined boundaries, could be introduced in control programs to identify neighborhoods at high risk for dengue transmission.

While a vaccine is under research, without immediate prospect for success, vector control remains the only way to prevent dengue transmission (*1*–*3*). Vector control programs are essentially based on source reduction, eliminating *Aedes aegypti* larval habitats from the domestic environment, with increasing community involvement and intersectoral action in recent decades (*4*,*5*). However, current entomologic indicators do not seem to reliably assess transmission risks, define thresholds for dengue epidemic alerts, or set targets for vector control programs (*6*,*7*). Therefore, defining new indicators for entomologic surveillance, monitoring, and evaluation are among the research priorities of the World Health Organization Special Programme for Research and Training in Tropical Diseases.

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Although only adult female *Aedes* mosquitos are directly involved in dengue transmission, entomologic surveillance has been based on different larval indices (*8*,*9*). The house index (HI, percentage of houses positive for larvae) and the Breteau index (BI, number of positive containers per 100 houses) have become the most widely used indices (*6*), but their critical threshold has never been determined for dengue fever transmission (*9*,*10*). Since HI≤1% or BI≤5 was proposed to prevent yellow fever transmission, these values have also been applied to dengue transmission but without much evidence (*8*,*11*). The Pan American Health Organization described 3 levels of risk for dengue transmission: low (HI<0.1%), medium (HI 0.1%–5%), and high (HI>5%) (*12*), but these values need to be verified (*13*). The vector density, below which dengue transmission does not occur, continues to be a topic of much debate and conflicting empiric evidence. For example, dengue outbreaks occurred in Singapore when the national overall HI was <1% (*14*). In contrast, researchers from Fortaleza, Brazil, found that dengue outbreaks never occurred when HI was <1% (*15*). However, different geographic levels are used to calculate the indices in the various studies, and the appropriated level for entomologic indices is in itself an issue of debate (*16*). Furthermore, the appropriateness of larval indices has been questioned; recently, as an alternative, pupal indices were developed by Focks et al. (*7*) to better reflect the risk for transmission. Still, their utility for source reduction programs is controversial, and the feasibility of pupal collection in routine *Aedes* surveillance is untested (*17*).

In this study, we assessed the usefulness of larval indices for identifying high-risk areas for dengue virus transmission. We examine the influence of measurements at different geographic levels, establish a threshold for epidemic outbreaks, and discuss their utility for community-based *Aedes* control programs.

Methods

Context

The Cuban dengue prevention program has been hailed as among the few success stories in *Aedes* control (*18*,*19*). It was initiated in 1981, during the first dengue hemorrhagic fever epidemic in the Americas (*20*). As a result of this effort, Cuba was free of dengue from 1982 to 1996, although *Aedes* was reported again from 1992 (*21*). In 1997, dengue transmission occurred in Santiago de Cuba, a municipality located in the eastern part of the country (*22*). The epidemic remained limited to this city, but *Aedes* mosquitoes were observed in 29 other municipalities, including Havana, the capital city, in the northwest of the country. After intensification of vector control activities in the entire country (*22*), HIs from 0.05% to 0.91% were observed in Havana between 1997 and 2001 (*23*). In spite of these low indices, an outbreak of 138 cases of dengue fever occurred in September and October 2000; both dengue 3 and dengue 4 viruses were isolated (*1*). Dengue serotypes 3 and 4 had never circulated in Cuba, and we can assume low or nonexistent immunity in the population. From June 2001 to February 2002, a new outbreak occurred, and 12,889 new dengue cases were confirmed (*23*).

Study Area

The study was conducted in Playa Municipality, in the northwest of Havana. The municipality has an area of 34.90 km^2 and a population of 182,485 inhabitants. It has an average annual temperature of 25°C and precipitation of 132.9 mm in the rainy season (May–October). The population density is 5,228 habitants per square kilometer. The municipality has a noncontinuous water supply (every 2 days) and irregular garbage collection. It is divided into 9 health areas, each providing primary care to ≈30,000 people. We performed an in-depth study in the 5 health areas where dengue transmission occurred in the September–October 2000 epidemic.

Study Design

We conducted a case-control study. Two units of analysis were used: blocks of houses (a block has on average 50 houses) and neighborhoods, which were defined as a block plus surrounding blocks (this definition generally results in clusters of 9 blocks with a radius of ≈100 m). These units are defined by manmade boundaries and not by ecologic determinants, per se, to usefully guide community-based control. We defined a "case" as a block (or neighborhood) of houses in the study area where ≥1 inhabitant was detected with confirmed dengue fever during the September–October 2000 outbreak. "Control" blocks (or neighborhoods) were randomly sampled from those in the study area where no dengue case was reported.

Data Collection

Dengue Fever

Dengue cases were defined as patients with fever and ≥2 symptoms of dengue fever such as myalgia, arthralgia, headache, and rash, with serologic confirmation by immunoglobulin M–capture enzyme-linked immunosorbent assay (*1*,*12*) at the national reference laboratory of viral diseases in the Institute of Tropical Medicine, Havana.

During the epidemic, suspected cases were identified through the health services. Additionally, a seroepidemiologic survey was conducted in the study area at the end of October 2000; all family physicians made home visits to families under their responsibility, searching for recent denguelike illnesses. Blood samples were collected from all persons with a history of fever.

All confirmed dengue patients (passively and actively found) were interviewed by their family physician, supervised by an epidemiologist of the health area, to determine the exact date of symptom onset and places visited in the 10 preceding days. The completeness of the collected information was verified by epidemiologists of the Institute of Tropical Medicine, and if necessary, patients were revisited.

Entomologic Information

We used entomologic surveillance data that were independently recorded by the National Vector Control Program. At 2-month intervals, vector control technicians exhaustively inspected every house in the Playa Municipality for larval stages of *Ae. aegypti*. We used data collected in 3 cycles, July–August 2000 (before the epidemic), September–October 2000 (during the epidemic), and November–December 2000 (after the epidemic). We extracted information on the number of inspected houses, positive containers (with *Ae. aegypti* pupae or larvae), and houses with ≥1 positive container. We eliminated 4.8% of the blocks from the study because they were not inspected in the 3 inspection cycles.

Data Analysis

We related all data collected to geographic coordinates by a unique house block code and introduced it in MapInfo software (MapInfo Corporation, Troy, NY, USA). Case-patients were located by their address in the corresponding block. For the 3 entomologic inspection cycles, HI and BI were calculated at the block, neighborhood, and health area level. Additionally, we identified the BI_{max}, which is the highest or maximum BI at the block level for each neighborhood of the case and control blocks included in the study. This variable is derived with the following equation:

$$BI_{\max} = \max_{\forall i \subset N} BI_i \ ,$$

where BI_i is the BI of the *i*th block belonging to the concerned neighborhood *N*, and $\forall i \subset N$ indicates that all BI_i of *N* are considered to identify the BI with the highest value as BI_{max}.

All data were exported to SPSS (SPSS Inc., Chicago, IL, USA) for analysis. We calculated the Spearman rank correlation coefficient between the different indices in the 3 inspection cycles. The entomologic indices were transformed to approximately normal distributions (by using square root transformation) for calculating means, standard deviations, and 95% confidence intervals. Differences in the distribution of the indices were assessed with the Mann-Whitney test.

We assessed the discriminative power of the indices by using receiver operating characteristic (ROC) curves. Their accuracy to discriminate between case and control blocks (and neighborhoods) was classified according to the value of the area under the ROC curve (AUC) (*24*) as noninformative (AUC≤0.5), less accurate (0.5<AUC≤0.7), moderately accurate (0.7<AUC≤0.9), highly accurate (0.9<AUC<1) and perfect (AUC = 1). The value of the indices with the highest sensitivity, >50% specificity, for discriminating case and control geographic units was taken as the optimal cutoff point. The lower limit of 50% specificity was set to safeguard positive predictive value and decrease the number of units falsely classified at high risk for dengue transmission, which triggers unnecessary action and generates unproductive costs. The association between the entomologic indices and dengue transmission was further explored by logistic regression models.

Results

During the epidemic, health services assisted 4,679 febrile patients in the 5 health areas included in the study. All patients were serologically tested 5 days after onset of fever, and dengue infection was confirmed in 47.

In the seroepidemiologic survey, 82.5% of the families were effectively visited by their family physician. The survey found 7,008 persons with symptoms of fever between September and October 2000 who had not previously attended the health services. Serum specimens were collected from all of them, and dengue infection was confirmed in 22.

As a result, 69 (47 passively identified plus 22 actively identified) dengue cases were confirmed, all patients were interviewed, and 4 cases epidemiologically related to outbreaks in other municipalities were excluded from the study. The final sample consisted of 65 confirmed dengue fever patients who lived in 38 different blocks in the 5 health areas included in the study.

In the July to August inspection cycle, before the outbreak, the overall municipal BI and HI were 0.92 and 0.87%, respectively (Table 1). The mean values of the indices calculated at the health area level were also ≈1 for areas with or without dengue cases during the subsequent epidemic. However, the mean BI and HI were >1 for case neighborhoods and substantially <1 for neighborhoods without cases. During the epidemic, the effect of the level of measurement of the indices was still more pronounced. The HI and BI at the municipality level were 1.53% and 1.73, respectively, but all health areas with dengue cases attained a BI >1. Even more marked differences existed at the block and neighborhood levels, and after the outbreak the indices returned to average values <1 at all levels of measurement. The mean values for case blocks and neighborhoods were, in all instances, consistently substantially and significantly higher (all $p<0.05$) than those for corresponding control units. A high correlation was observed between block-level BI and HI values ($r \geq 0.94$, $p<0.05$). In most positive houses (89.6%), only 1 container with *Aedes* larvae or pupae was found.

The Figure shows the spatial distribution of *Ae. aegypti* larval infestation during the inspection cycles before, during, and after the epidemic and the location of the dengue fever cases in the first (September) and second (October) month of dengue virus transmission. In most blocks (70%), no *Aedes* infestation was present before the epidemic period, but 8.8% of blocks had BI values >4, with a maximum BI of 50. Of the 17 confirmed dengue patients in September, only 3 (18%) lived in a block with BI≥4 in the July–August inspection cycle. However, 15 (88%) lived in a neighborhood with at least 1 block with BI≥4. The *Aedes* infestation increased during the second inspection cycle and then decreased again, concurrent with the intensified vector control activities during the epidemic. From November to December, after the outbreak, 71.6% of house blocks were *Aedes*-free, while 6.3% had BI>4.

The mean block BI, the mean neighborhood BI, and the mean BI_{max} for case and control blocks are given in Table 2. Before the epidemic, the mean BI values were approximately equal for case and control units. However, the BI_{max} values were significantly higher for neighborhoods of case blocks. While transmission started in neighborhoods with high BI_{max} infestation levels, it spread into blocks and neighborhoods with lower mean BI values in October. Still, during the epidemic, the indices remained systematically and significantly higher in case blocks. After the epidemic, they returned to similar values for case and control units.

The entomologic indices from inspection cycles before and during the epidemic were less to moderately accurate at predicting subsequent transmission. The highest AUC value, 0.71, was attained with the BI_{max} from the July to August inspection cycle. At the cutoff of 4.07, it reached a sensitivity of 77.8% and a specificity of 63.2% for

Table 1. Mean house index (HI) and Breteau index (BI) before, during, and after the dengue outbreak and mean area and population at different geographic levels, Playa Municipality, Havana, 2000

	July–August 2000 (before outbreak)		September–October 2000 (during outbreak)		November–December 2000 (after outbreak)			
Level	HI (%)	BI	HI (%)	BI	HI (%)	BI	Area (km^2)	Population
Municipality	0.87	0.92	1.53	1.73	0.69	0.73	34.90	182,485
Health area*								
With cases (n = 5)	0.92	0.99	1.97	2.34	0.48	0.50	2.85	21,815
Without cases (n = 4)	1.03	1.08	0.89	1.06	0.87	0.93	5.13	16,320
Neighborhood†								
With cases (n = 38)	1.12	1.12	4.00	4.53	0.80	0.84	0.078	2,057
Without cases (n = 38)	0.64	0.69	1.39	1.52	0.74	0.81	0.062	1,466
Block†								
With cases (n = 38)	0.33	0.34	2.40	2.92	0.62	0.66	0.010	271
Without cases (n = 38)	0.13	0.20	0.35	0.42	0.32	0.33	0.008	195

*For all areas in the municipality.
†For neighborhoods/blocks included in the study.

predicting September transmission. A neighborhood BI≥1.30 gave similar results. Block-level BIs were less accurate. Comparable cutoff points for the indices in the September to October inspection cycle discriminate best for predicting transmission in October (data not shown). After the epidemic, in the November to December inspection cycle, the indices had a high specificity: 89.6% for BI<1 and 85.7% for BI_{max}<4, which points toward their usefulness in nonepidemic periods.

Table 3 shows the odds ratios (OR) for dengue transmission at optimal BI cutoff values. From July to August, consistent with previous results, only BI_{max}≥4 was a significant predictor for identifying blocks with a case in September (OR 6.00, p<0.05). In contrast, the OR for all the different September–October BIs were significant; blocks above threshold had 3–5 times the chance of having a dengue case in October. Additionally, during the outbreak, the presence of a single positive container in a block was associated with a higher risk for dengue transmission (OR 3.49, p<0.05).

Discussion

We show that entomologic indices, BI in particular, allow identification of geographic units at high risk for dengue transmission. However, in regions with low *Ae. aegypti* density, identifying such units requires analysis at different levels, i.e., for blocks and neighborhoods, and short intervals between inspection cycles. Optimal cutoff values were identified for our study setting.

The existence of detailed surveillance data before, during, and after the dengue epidemic in Playa Municipality offered a unique opportunity to analyze entomologic information at different geographic levels. Entomologic data collected through routine systems, however, has some limitations. First, larval prevalence was possibly slightly underestimated: blocks were inspected by different vector control technicians, procedures used may not have been completely standardized, and few data are (randomly) missing. Second, when dengue cases were reported, the control program intensified, and more *Aedes* foci may have been detected. Third, sampling *Aedes aegypti* can be time sensitive (*25*), and our inspection cycles at 2-month intervals may not have fully captured the temporal variability of the entomologic indices. Besides, we may not have been able to identify all dengue patients who were

Figure. Spatial distribution of dengue cases and Breteau indices (BI) at the block level before, during, and after the dengue outbreak, Playa Municipality, Havana, 2000.

Table 2. Mean BI for case and control blocks before, during, and after the dengue outbreak, Playa Municipality, Havana, 2000*

	July–August 2000 (before epidemic), mean (95% CI)			September–October 2000 (during epidemic), mean (95% CI)			November–December 2000 (after epidemic), mean (95% CI)		
Block	BI	NBI	BI_{max}	BI	NBI	BI_{max}	BI	NBI	BI_{max}
September case blocks (n = 9)	0.53 (0.02–1.75)	1.52 (0.76–2.53)	6.28† (3.29–10.23)	11.95† (2.26–29.27)	10.75† (6.73–15.70)	28.4† (16.1–44.1)	0.63 (0.04–1.70)	0.64 (0.37–0.91)	2.94 (1.71–4.83)
October case blocks (n = 29)	0.29 (0.05–0.72)	1.01 (0.60–1.54)	4.24 (2.48–6.46)	1.39† (0.50–2.71)	3.16† (1.99–4.61)	12.2† (7.79–17.6)	0.66 (0.06–0.91)	0.76 (0.44–1.06)	2.87 (1.50–4.35)
Control blocks (n = 38)	0.20 (0.02–0.58)	0.69 (0.42–1.02)	2.96 (1.71–4.56)	0.42 (0.07–1.05)	1.52 (0.91–2.29)	1.52 (3.57–8.32)	0.33 (0.06–0.82)	0.68 (0.36–1.18)	2.34 (1.43–4.27)

*BI, Breteau index; CI, confidence interval; NBI, neighborhood BI; BI_{max}, maximum BI at the block level for each neighborhood.
†Significantly different from corresponding values for control blocks ($p<0.05$).

infected outside their area of residence. Also, the study design did not allow us to detect asymptomatic dengue infections, which likely occurred in some control blocks and neighborhoods. However, we expect the potential misclassification to be nondifferential, i.e., independent of the entomologic indices. Furthermore, the experience of the technicians of the vector control program, their close supervision (including systematic revisiting of 33.3% of the inspected houses), and the interviews conducted with all dengue patients to exclude outside infection guarantee that biases, if any, are minimal.

Various researchers have investigated the relationship between dengue transmission and the *Aedes* population, expressed as larval (*15*,*26*–*31*), pupal (*7*,*13*,*32*), and adult indices (*33*). Moore (*28*) in Puerto Rico and Pontes (*15*) in Fortaleza, Brazil, used temporal graphics to compare the seasonal fluctuation of rainfall, *Aedes* larval indices, and dengue incidence. They observed a strong relation in the patterns of the 3 series. In Puerto Rico, the peak incidence of confirmed infection followed the peak larval density by ≈1 month. In Salvador, Brazil, sentinel surveillance in 30 areas detected a significant 1.4× higher seroincidence when the HI was >3% (*31*). Recently, Scott and Morrison (*16*) showed that traditional larval indices in Peru are correlated with the prevalence of human dengue infections. The variety of thresholds proposed in these and other studies could be partially explained by different methods and geographic levels of analysis used, but other factors influence the relationship between *Aedes* density and transmission risk, such as herd immunity (*11*), population density (*31*), mosquito-human interaction (*34*), virus strain, and climate, which affects mosquito biology and mosquito-virus interactions (*16*).

Table 3. OR for dengue transmission at the optimal cutoff values of the BI, Playa Municipality, Havana, 2000*

Index and cutoff value†	OR (95% CI)
July–August 2000 inspection cycle (before epidemic)	
BI per block >0	
September transmission	2.57 (0.57–11.70)
October transmission	1.69 (0.58–4.94)
BI per neighborhood ≥1	
September transmission	3.00 (0.66–14.17)
October transmission	1.08 (0.40–2.90)
BI_{max}≥4	
September transmission	6.00 (1.09–32.98)‡
October transmission	1.21 (0.45–3.25)
September–October 2000 inspection cycle (during epidemic)	
BI per block >0	
October transmission	3.49 (1.20–10.10)‡
BI per neighborhood ≥1	
October transmission	5.06 (1.46–17.38)‡
BI_{max}≥4	
October transmission	3.44 (1.23–9.63)‡

*OR, odds ratio; BI, Breteau index; CI, confidence interval; BI_{max}, maximum BI at the block level for each neighborhood.
†Optimal cutoff value determined as specified in Methods.
‡$p<0.05$.

Entomologic indices, however, were strongly associated with transmission, and we used ROC analysis (*24*) to assess the potential of these indices to predict in which blocks transmission would occur and to select an operating point that would provide an optimum tradeoff between false-positive and false-negative results (*35*). BI_{max}≥4 followed by neighborhood BI≥1 during the preceding ≈2 months provides good predictive discrimination. At longer intervals, the sensitivity of these indices becomes too low. More frequent inspection cycles might perform better since *Aedes* needs only 9–12 days to develop from egg to adult (*36*). Care should, however, be taken when extrapolating these findings to communities with other herd immunity levels or different environmental conditions.

Our data also show that the geographic level of analysis determines the *Aedes* indices obtained. Marked heterogeneity is not only found inside Playa Municipality but also inside smaller health areas. Indices at the neighborhood level perform best, followed by indices at the block level. Geographic scale has too often been neglected when dengue transmission is studied. In general, overall indices are calculated for communities (sometimes of different sizes) defined by administrative boundaries, which do not constitute entomologically homogeneous units. Notwith-

standing, local variability of larval indices can be inferred from the literature, in which it is sometimes mentioned. Chan et al. (*27*) noted that HI in different sections of Singapore's Chinatown varied from 10.2% to 25.0%. However, Goh et al. (*30*) reported an overall HI of 2.4% in Singapore, but at the level of 7 blocks taken together (approximately the same scale as our neighborhood), HI up to 17.9% were found. Tran et al. (*36*) defined 400 m and 40 days as the spatial and temporal boundaries of maximum dengue transmission in a dengue focus. Perez et al. (*37*) identified areas in Havana with heterogeneous risks for vector infestation by using a geographic information system. Spatial heterogeneity has also been observed at the household level for both *Aedes* populations (*10*,*38*,*39*) and dengue transmission (*26*,*29*,*40*), but this level seems less suitable for identifying areas for intervention. Blocks or neighborhoods, given the epidemiologic situation in our study area, are a more appropriate scale.

The unit of analysis used in our study, the block, is based on manmade boundaries. While these may not describe the ecology of risk, they seem to be useful markers from the perspective of community-based control interventions. In most settings, appropriately sized and locally meaningful geographic units could be similarly defined for entomologic surveillance, but the use of different boundaries or different analytical techniques could produce different results.

In our study, BI≥1 and BI_{max}≥4 seemed to be a suitable action threshold and target, respectively, in community based dengue prevention. However, these results are derived from the analysis of 1 epidemic, and the thresholds identified may not constitute suitable targets in another epidemic or in locations where different ecologic conditions prevail. Similar studies in future epidemics and in other settings are necessary to verify the general applicability of our results.

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Enterobacter cloacae Outbreak and Emergence of Quinolone Resistance Gene in Dutch Hospital

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An outbreak of *Enterobacter cloacae* infections with variable susceptibility to fluoroquinolones occurred in the University Medical Center Utrecht in the Netherlands in 2002. Our investigation showed that a *qnrA*1 gene was present in 78 (94%) of 83 outbreak isolates and that a *qnrA1*-encoding plasmid transferred to other strains of the same species and other species. The earliest isolate carrying this same plasmid was isolated in 1999. *qnrA1* was located in a complex integron consisting of the *intI1*, *aadB*, *qacEΔ1*, *sul1*, *orf513*, *qnrA1*, *ampR*, *qacEΔ1*, and *sul1* genes that were not described previously. On the same plasmid, 2 other class 1 integrons were present. One was a new integron associated with the $bla_{CTX\text{-}M\text{-}9}$ extended-spectrum β-lactamase.

Multidrug-resistance among *Enterobacteriaceae*, including resistance to quinolones, is increasing. Although quinolone resistance is predominantly caused by chromosomal mutations, it may also result from a plasmid-encoded *qnr*-gene (*1*). The QnrA determinant, a 218–amino acid protein, protects DNA gyrase and topoisomerase IV from the inhibitory activity of quinolones (*2*). However, expression of *qnrA* alone is frequently insufficient to reach Clinical and Laboratory Standards Institute breakpoints for ciprofloxacin resistance. Since first identified in 1994 in the United States, *qnrA*-like genes have been sporadically identified in Enterobacteriaceae worldwide (*3*–*9*).

At the end of 2002, an outbreak of aminoglycoside-resistant *Enterobacter cloacae* infections with variable susceptibility for ciprofloxacin was detected in the University Medical Center Utrecht (UMCU), the Netherlands, involving >80 patients (*10*). The first aim of this study was to test the hypothesis that the variable susceptibility to ciprofloxacin of the outbreak strain was associated with plasmid-mediated *qnrA* and if so, to characterize the gene's molecular background and determine its ability to transfer in vitro as well as in vivo. Maximum circumstantial evidence for horizontal transfer in vivo with the outbreak strain as donor would be obtained if the following observations were made: 1) different species or strains collected from the same patient harbored the same *qnrA*-encoding plasmid; 2) this same *qnrA*-encoding plasmid was not found in patients without an epidemiologic link to the outbreak. The second aim of this study was to determine to what extent the *qnrA* gene is an emerging resistance problem in our hospital.

Materials and Methods

Bacterial Isolates

A total of 1,167 isolates were tested for a *qnrA* gene. Group I consisted of 178 *E. cloacae* pulsed-field gel electrophoresis (PFGE) typed isolates obtained from January 2001 to August 2003 from 159 patients (*10*). Of these, 83 tobramycin-resistant isolates obtained from 83 patients belonged to 1 clonal lineage (cluster I, outbreak strain). Five of these patients also carried a tobramycin-susceptible variant of the clonal lineage (I^A). The remaining 95 *E. cloacae* isolates contained 5 small clusters of 2 isolates each (III–VII), 1 cluster with 6 isolates (VIII), 1 cluster with 3 isolates (II), and 70 unique strains.

Groups II and III consisted of aminoglycoside-resistant, gram-negative bacteria identified in the hospital database that were other than the outbreak strain; these bacteria were isolated from patients with an outbreak strain (group II) as well as from patients not involved in the outbreak but admitted in the same period (January 2001–August 2003) (group III). Aminoglycoside resistance was the selection criterion because the outbreak strain was aminoglycoside-

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resistant, and these isolates are stored routinely in our laboratory. Group IV consisted of 867 *Enterobacteriaceae* isolates comprising 8 different species collected from 3 different origins: 269 clinical isolates from UMCU (1994–2000), 514 isolates from 23 European hospitals (1997–1998), and 84 fecal screening isolates from 53 patients at admission at UMCU (2000) (*11*).

Identification and Susceptibility Testing

Identification and susceptibility testing of isolates obtained through 2000 were performed by using the VITEK1 System with AMS R09.1 software (bioMérieux, Marcy-L'etoile, France); isolates obtained after 2000 were tested by using the Phoenix 100 Automated Microbiology System version V3.22 software (Becton Dickinson Biosciences, Sparks, MD, USA). For susceptibility testing, Clinical and Laboratory Standards Institute guidelines were used (*12*). In the conjugation experiments, MICs were determined by using Etest (AB Biodisk, Solna, Sweden).

Genotyping and Characterization of β-Lactamases

E. cloacae isolates were typed by PFGE. *Citrobacter freundii*, *Escherichia coli*, and *Klebsiella pneumoniae* were typed by PFGE and random amplified polymorphic DNA (*13*). To determine the kind of β-lactamases the outbreak strain expressed, isoelectric focusing (IEF) was performed with Phastgels (pH gradient 3–9) with the PhastSystem (Pharmacia AB, Uppsala, Sweden) (*14*). β-lactamases of isoelectric pH (pI) 5.6 (TEM-1), pI 7.6 (SHV-2A), and pI 8.2 ($bla_{\text{CTX-M-9}}$) and a broad range pI calibration set (Amersham Biosciences, Little Chalfont, UK) were used. β-lactamases were detected with nitrocefin (Oxoid, Basingstoke, UK).

Detecting and Characterizing Resistance Genes

Target DNA for polymerase chain reaction (PCR) assays was extracted by heating bacterial suspensions for 10 min at 95°C. *qnrA*, $bla_{\text{CTX-M}}$, and *aadB* were detected by PCR with primers and annealing temperatures described in the Table. The outbreak strain carried an integron containing an *aadB* gene encoding aminoglycoside resistance (*17*). Primers were developed to detect *aadB* gene and the downstream 3′-conserved segment (CS) of the integron in the same PCR (*aadB*-3′CS). PCR assays were performed for 30 or 35 cycles. The AmpC PCR tests were performed as described earlier, except that a single PCR format was used (*18*).

The $bla_{\text{CTX-M}}$ gene from *E. cloacae* 02-477 was sequenced by using CTX-M-9 group sequence primers (Table). The flanking regions of the *qnrA* gene and the $bla_{\text{CTX-M-9}}$ gene were determined by using a PCR and DNA sequencing strategy based on the sequences from In7, In36, In37, In60, and an integron from *E. coli* O159 (*5*,*9*,*19*–*22*). To confirm that the gene cassettes were part of a complex integron with *qnrA* or $bla_{\text{CTX-M-9}}$, we used the Expand Long Template PCR system (Roche, Woerden, the Netherlands) that employed primers to amplify sequences between the *qnrA* or $bla_{\text{CTX-M-9}}$ and the possible gene cassettes. All amplified products were (partly) sequenced for confirmation. Sequencing was performed with Qiagen Quick (Qiagen, Westburg b.v., Leusden, the Netherlands) purified PCR products by using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit and a 3100 capillary DNA sequencer (Applied Biosystems, Nieuwerkerk a/d Yssel, the Netherlands).

Conjugation Experiments

For conjugation experiments, an *E. coli* K12 and a tobramycin-susceptible clinical *E. cloacae* (03-702) isolate of PFGE cluster I^A were used as recipients. An *E. cloacae* (02-477) belonging to PFGE cluster I was used as donor. Conjugation was performed as described (*23*). MacConkey agar plates containing tobramycin (8 μg/mL) were used for counter selection, and transconjugants were selected on colony form. Conjugation was confirmed by a *qnrA*-specific PCR. Secondly, transconjugant *E. coli* C02-477A was used as a donor for *qnrA*-negative *E. cloacae* 03-702 belonging to cluster I^A. Transconjugants were selected by using 15 μg/mL ampicillin-clavulanic acid and 5 μg/mL tobramycin. Transconjugants were characterized as described above.

Detecting Resistance Genes on Plasmid by Southern Hybridization

Plasmids were isolated with the Qiagen Plasmid Maxi Kit (Qiagen). Plasmid DNA was separated on 1% PFGE agarose (Bio-Rad Laboratories, Richmond, CA, USA) in 0.5× Tris-borate-EDTA, 0.05 mmol/L thiourea buffer at 14°C in CHEF DR-II apparatus (Bio-Rad). Run time was 22 h with a voltage of 6 V/cm and a linearly ramped pulse time of 30 to 70 s. The DNA was blotted and hybridized. The probes were PCR amplification products obtained with primers used to detect *aadB*-3′-CS, $bla_{\text{CTX-M-9}}$, and *qnrA* genes (Table). Products were labeled with the AlkPhosDirect Reaction Kit (Amersham Biosciences) and detected with CPD-Star (Amersham Biosciences).

Results

qnrA1 in Outbreak Strain

For 78 (94%) of the 83 *E. cloacae* isolates in cluster I (outbreak strain), the *qnrA*-specific PCR was positive. To confirm results from the PCR, 2 fragments were sequenced. The obtained sequences were identical to the published sequence of *qnrA1* (GenBank accession no. AY070235).

Table. Oligonucleotides used for polymerase chain reaction amplification and sequencing

Target	Primer	5′–3′ sequences	GenBank accession no.	Nucleotide positions	Annealing temperature (°C)	Amplicon size (bp)	Source
QnrA	qnrAR	AGG AAG CGC CGC TGA GAT TG	AY070235	762–743	56	281	This study
	qnrAF	CTA TGC CGA TCT GCG CGA TG	AY070235	482–501			This study
aadB-3′CS	aadB	TGG AGG AGT TGG ACT AT	AY173047	251–267	55	432	This study
	3′CS	AAG CAG ACT TGA CCT GA	M73819	1342–1326			(*15*)
bla_{CTX-M-}: most	ctx-m-uni-F	CGA TGT GCA GTA CCA GTA A	U95364	214–232	50	538	This study
	ctx-m-uni-R	ATA TCG TTG GTG GTG CC	U95364	751–735			This study
bla_{CTX-M-}: 2,4,5,6,7, 20,Toho-1	ctx-m-2F	ATG ATG ACT CAG AGC ATT CG	X92507	6–25	58	884	(*16*)
	ctx-m-2R	TTA TTG CAT CAG AAA CCG TG	X92507	889–870			(*16*)
bla_{CTX-M-}: 3,10,11,12, 15,22,25	ctx-m-10-1F	ATG GTT AAA AAA TCA CTG CG	X92506	63–82	60	872	This study
	ctx-m-10-4R	AAA CCG TTG GTG ACG AT	X92506	934–918			This study
bla_{CTX-M-}: 9,13,14,15,16,17,18, 19,24, Toho-2 and –3	ctx-m-9F	AGA CGA GTG CGG TGC AGC AA	AJ416345	217–236	67	773	This study
	ctx-m-9R	GAT TCT CGC CGC TGA AGC CA	AJ416345	989–970			This study
Sequence $bla_{CTX-M-9}$ group	ctx-m-9-1F	TGG TGA CAA AGA GAG TGC AAC G	AJ416345	133–154			This study
	ctx-m-9-MF	GGA GGC GTG ACG GCT TTT	AJ416345	576–593			This study
	ctx-m-9-MR	AAA AGC CGT CAC GCC TCC	AJ416345	593–576			This study
	ctx-m-9-4R	TCA CAG CCC TTC GGC GAT	AJ416345	1007–990			This study

Susceptibility testing showed that 87% of the 83 outbreak isolates were resistant or intermediate resistant to ciprofloxacin (43% resistant, 43% intermediate resistant), 100% were resistant to tobramycin, 63% to gentamicin, 2% to amikacin, 100% to ceftriaxone, 12% to trimethoprim-sulfamethoxazole, and 0% to carbapenems. A total of 81 (98%) of the 83 isolates harbored an *aadB* containing integron.

IEF showed the presence of a β-lactamase with a pI of ≈8.2, which suggested the presence of either an AmpC β-lactamase or a CTX-M type extended-spectrum β-lactamase. No AmpC-specific amplification products were obtained. Eighty-two (99%) of the 83 isolates harbored a bla_{CTX-M} gene. DNA sequencing showed the presence of $bla_{CTX-M-9}$.

The plasmid (pQC) of conjugant *E. coli* C02-477A was isolated, and its size was estimated at 180 kb by agarose gel electrophoresis. Southern blotting that used specific probes confirmed that pQC contained the *qnrA1* gene, the $bla_{CTX-M-9}$ gene, and the integron with an *aadB* gene cassette (data not shown). Sequences flanking the *qnrA1* and $bla_{CTX-M-9}$ genes were comparable with 3 previously described class 1 integrons (Figure 1). The first integron (In-UMCU-1 accession no. AY987395), containing the *qnrA1* gene, had the same additional structures as In36, *orf513*, *qnrA1*, *ampR*, plus a second copy of the 3′-conserved segment. The In36 integron contained the gene cassettes *drf16* and *aadA2*, while In-UMCU-1 contained only the *aadB* gene cassette. In addition, the DNA sequences between the second s*ul1* gene and o*rf5* (bp 9606–9624 of In36) differed from the sequence of In-UMCU-1 (*5*). The second integron (In-UMCU-2, accession no. DQ108615), which contained $bla_{CTX-M-9}$, was comparable to In60, but In60 contained the *drf16* and *aadA2* gene cassettes, while In-UMCU-2 contained the *aadB* gene cassette (*21*). The third integron (In-UMCU-3, accession no. DQ019420), which contained the gene cassettes *sat*, *psp*, and *aadA2*, was described previously in an enterotoxigenic *E. coli* O159 isolated in Japan (*22*). PCR amplification of the *aadA2* gene of the donor, recipient, and transconjugants indicated that this third integron was also located on pQC.

Evidence for Transfer of qnrA in vitro

In vitro conjugation experiments showed that pQC could be transferred both from and to the outbreak strain (Figure 2). pQC was successfully transferred from

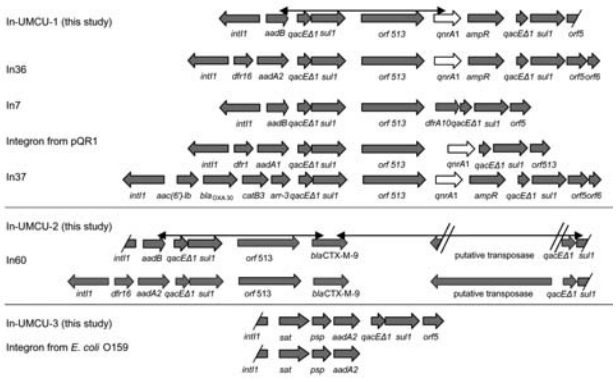

Figure 1. Schematic presentation of integrons on pQC compared with previously described integrons (*5,9,19,21,22*). The black double-pointed arrows indicate the product amplified with an Expand Long Template PCR system (Roche, Woerden, the Netherlands), demonstrating a link between the *qnrA* and $bla_{CTX\text{-}M\text{-}9}$ genes and their respective integrons.

E. cloacae 02-477 to recipient *E. coli* K12. The resulting transconjugant *E. coli* was subsequently used as donor to transfer pQC to a type I^A *E. cloacae* (03-702), which resulted in a successful transfer of pQC. pQC conferred increased ciprofloxacin MICs (from 6- to 10-fold) and resistance to tobramycin, tetracycline, and ceftriaxone to the transconjugants (Figure 2). Acquisition and loss of pQC were associated with 2 changes in the PFGE pattern.

Evidence for Transfer of *qnrA1* in vivo

Different species or strains collected from the same patient harbored the same pQC. From 22 of the 53 patients with an outbreak strain, 35 other tobramycin-resistant, gram-negative clinical isolates were available. Eleven different strains obtained from 11 patients were positive for *qnrA1*, $bla_{CTX\text{-}M\text{-}9}$, and *aadB*-3′-CS. These comprised 4 different species: *C. freundii* (n = 1), *Enterobacter aerogenes* (n = 1), *E. coli* (n = 7), and *K. pneumoniae* (n = 2). Plasmid isolation from 6 *E. coli* and 1 *K. pneumoniae* yielded a plasmid of the same size as the pQC in the outbreak strain. Because of its large size and possibly a very low copy number, only small amounts of plasmid DNA could be isolated. These amounts were insufficient to perform further comparative analyses by restriction fragment analysis or Southern blotting.

Some *E. cloacae* strains with a strong epidemiologic link to the outbreak strain were also pQC positive. All isolates belonging to clusters III, VII, and VIII contained pQC as well as 5 *E. cloacae* isolates with a unique genotype. Plasmid isolation of 3 strains again showed a plasmid of the same size as the outbreak pQC. Three of the 5 unique isolates were obtained from patients who also harbored the outbreak strain.

The *qnrA* gene, the *aadB*-containing integron, and the $bla_{CTX\text{-}M\text{-}9}$ could not be detected in PFGE cluster I^A, which is closely related to the outbreak strain (Figure 3). The loss of these genes was associated with increased susceptibility to ciprofloxacin, tobramycin, ceftriaxone, and tetracycline. In addition, an identical change in the PFGE pattern was observed, as in the in vitro experiments. These results suggest that the host may lose pQC in vivo.

qnrA1 Recent Emergence as Clinical Problem

pQC was not found in isolates obtained from patients without an epidemiologic link to the outbreak. No *qnrA1* gene was detected in any of 83 aminoglycoside-resistant gram-negative organisms (44 *E. coli*, 19 *K. pneumoniae*, 4 *Proteus mirabilis*, 2 *Klebsiella oxytoca*, 2 *E. cloacae*, 1 *Enterobacter* sp., 7 *C. freundii*, 4 *Serratia marcescens*) obtained from 74 patients admitted to wards not involved in the outbreak during the outbreak period. Neither was *qnrA*1 detected in any of the 269 UMCU isolates or the 84 community isolates.

Only 1 *qnrA1*-positive isolate was found in the 514 European isolates. This *qnrA1*-positive isolate was an *E. cloacae* organism isolated in 1999 at a surgical ward at UMCU that belonged to cluster III. The other 2 cluster III isolates were isolated at the same surgical ward during the outbreak period.

Discussion

We report a nosocomial outbreak with an R-plasmid–encoded *qnrA1* gene. This plasmid (pQC) was first detected in an *E. cloacae* isolated in 1999 and subsequently in another *E. cloacae* strain that caused a large outbreak in our hospital, starting in 2001. Strong evidence is provided that this outbreak strain was the source from which pQC disseminated to other strains of the same species and other species by horizontal gene transfer. The *qnrA1* gene was not detected in any of the hospital isolates (1994–2003) tested without an epidemiologic link to the

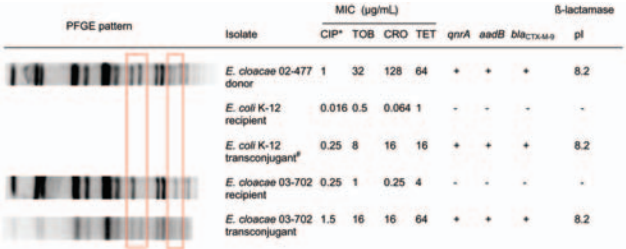

Figure 2. Pulsed-field gel electrophoresis (PFGE) patterns, susceptibility patterns, and key resistance genes for recipient and transconjugants in in vitro conjugation experiments. Boxes denote the area of variability in the PFGE patterns between isolates with and without pQC. CIP, ciprofloxacin; TOB, tobramycin; CRO, ceftriaxone; TET, tetracycline. **Escherichia coli* transformant served as donor for *Enterobacter cloacae* 02-0702.

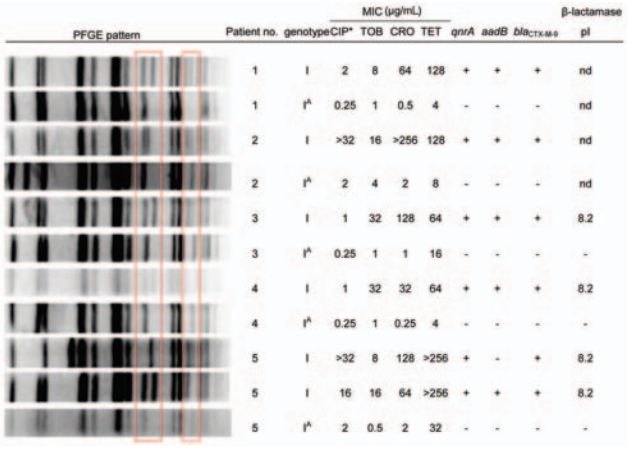

Figure 3. Pulsed-field gel electrophoresis (PFGE) patterns, susceptibility patterns, and key resistance genes of *Enterobacter cloacae* isolates from patients harboring isolates belonging to genotype I as well as I^A. Boxes denote the area of variability in the PFGE patterns between isolates with and without pQC. CIP, ciprofloxacin; TOB, tobramycin; CRO, ceftriaxone; TET, tetracycline.

outbreak strain, indicating that *qnrA1* is a new emerging resistance trait in our hospital.

pQC contained 3 different class 1 integrons. One integron was identical to an integron detected in an *E. coli* O159 from Japan (*22*). The 2 other integrons were complex integrons, In-UMCU-1 and In-UMCU-2, which were not described previously. Complex integrons are composed of a 5′-CS, gene cassettes, 3′-CS, *qacΔE*, *sulI*, additional genes, *qacΔE*, and *sulI*. These additional genes differ from gene cassettes by lacking a 59-bp element and having their own promoter (*24*). The *qnrA1* gene in In-UMCU-1 was also present as an additional gene, as was the case for the 3 previous characterized *qnrA1* genes in In36, In37, and the complex integron of pQR1 (*5*,*9*). The sequences of these genes were identical for In-UMCU-1, In 36, and In37, and slightly different for the integron on pQR1. The gene cassette content of the 4 integrons, however, was different, although all 4 possessed a gene encoding aminoglycoside resistance. All *qnrA1*-positive isolates reported in the literature also show resistance to cephalosporins (*1*,*4*–*9*,*25*–*27*). Therefore, *qnrA1* seems to be closely associated with resistance to cephalosporins and aminoglycosides. How these comparable but different complex integrons arose is unclear. Either the same additional genes became associated with different integrons or the gene cassettes in an original complex integron were exchanged.

Our study confirmed previous findings that the presence of *qnrA1* does not necessarily lead to MICs above Clinical and Laboratory Standards Institute breakpoints for resistance to ciprofloxacin (*1*,*3*,*7*,*25*). Therefore, the presence of *qnrA1* had no therapeutic consequences for the patients from whom these isolates were obtained. However, the increased MIC may provide the host bacterium a selective advantage in an environment of low concentrations of quinolones, increasing the bacterial numbers and therefore the absolute chance of a chromosomal mutation encoding resistance (*7*,*25*). The presence of a *qnrA*-carrying plasmid might even enhance the mutation rate encoding quinolone resistance (*1*). Furthermore, acquisition of *qnrA* by a host bacterium that already contains quinolone resistance mechanisms may raise MICs above the LCSI breakpoints (*25*,*28*,*29*). As shown in this study, the same plasmid may cause fluctuation in susceptibility in MICs in different recipients because of variation in porin expression or mutations in the gyrase or efflux pump-encoding genes (*2*).

In conclusion, in a hospital setting the *qnrA* gene is advantageous for the host bacterium. Because of this gene's location on promiscuous R-plasmids, it is likely to emerge worldwide.

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Mr Paauw is a doctoral candidate at the Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation, UMCU, the Netherlands. His research is focused on detecting and characterizing genetic features that can enhance virulence, resistance, and epidemic behavior of Enterobacteriaceae.

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Spatial Analysis of Sleeping Sickness, Southeastern Uganda, 1970–2003

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Sleeping sickness reemerged in southeastern Uganda in the 1970s and remains a public health problem. It has continued to spread north into new districts, and gaps remain in the understanding of the causes of its spread and distribution. We report the distribution and magnitude of sleeping sickness in southeastern Uganda from 1970 to 2003. Data were collected from records of the Ugandan Ministry of Health, individual sleeping sickness treatment centers, and interviews with public health officials. Data were used to develop incidence maps over time, conduct space-time cluster detection analyses, and develop a velocity vector map to visualize spread of sleeping sickness over time in southeastern Uganda. Results show rapid propagation of sleeping sickness from its epicenter in southern Iganga District and its spread north into new districts and foci.

Sleeping sickness is the human form of African trypanosomiasis (caused by *Trypanosoma* spp.), a protozoal parasitic disease affecting humans, livestock, and many sylvatic species in sub-Saharan Africa. It is transmitted by the tsetse fly vector (*Glossina* spp.) and in cattle is a serious constraint to livestock development in sub-Saharan Africa (*1*–*3*).

The acute form of sleeping sickness, which is caused by *Trypanosoma brucei rhodesiense* and is predominant in eastern and southern Africa (*4*–*6*), is present in southeastern Uganda (Figure 1). Sleeping sickness is a serious public health problem in this region; epidemics have occurred in 1901–1915, 1940–1946, and 1976–1989 (*3*). More recently, spread of sleeping sickness into areas previously thought to be free from the disease has highlighted gaps in the ability of current research to explain and predict the distribution of infection (*7*).

In 1976, an outbreak was detected in Luuka County in western Iganga District, outside the traditional fly zone. This was the beginning of an extensive epidemic that eventually spread throughout southeastern Uganda. This outbreak occurred during a time of great political instability and civil conflict in Uganda, which contributed to a reduction of resources and services for sleeping sickness (*8*). Although the incidence of sleeping sickness decreased in southeastern Uganda in the early 1990s, it continues to persist and spread in 2005. An outbreak was detected for the first time in Soroti District in 1998 (*7*), followed by

Figure 1. Location of the study site in southeastern (SE) Uganda. The star indicates the capital of Kampala. Inset shows surrounding countries in Africa.

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continued spread north into Kumi, Kaberamaido, and Lira Districts (*9*,*10*). Historical analyses of sleeping sickness in southeastern Uganda can improve disease control by increasing understanding of the context and trends of the disease, as well as identifying variables associated with these trends. Additionally, historical analyses may validate hypothesized processes.

We describe and characterize the spatial distribution of *T. b. rhodesiense* sleeping sickness in southeastern Uganda for a 34-year epidemic period (1970–2003). We hypothesize that sleeping sickness in southeastern Uganda is driven by 2 dominant processes. In process A, in regions where disease occurs or has recently occurred, localized outbreaks are triggered by processes that increase tsetse populations or by changes that increase human-tsetse contact. In process B, in regions where disease has not recently occurred, spread is facilitated by movement of infected livestock into uninfected regions.

Methods

Study Area

The study area in southeast Uganda in eastern Africa (Figure 1) is subdivided into 17 districts, 46 counties, and 254 subcounties. The region has an area of ≈55,000 km^2 and a population of ≈9 million (*11*). Thirteen percent live in the capital of Kampala (*11*), and the remainder live in predominantly rural areas dominated by livestock and subsistence farming (*12*,*13*).

Data Collection

Cross-sectional sleeping sickness data from 1970 to 2003 were collected retrospectively in 2004 to identify case counts and measures of disease magnitude per subcounty per year. Data were collected for all available records of sleeping sickness patients in southeastern Uganda. Data availability and reliability varied between years on the basis of quality of surveillance and primary data collection, as well as availability of records and recall bias for secondary data collection. Reliability of data for 1986 to 2003 was considered moderate to high, but reliability of data for 1970 to 1986 was low to moderate. Evaluation and review of data by public health officials concluded that information on disease prevalence and absence was reliable for most years, but measures of disease magnitude were less reliable before 1986.

Since 1999, sleeping sickness data summaries have been provided by the National Sleeping Sickness Control Program at the Ugandan Ministry of Health. For data before 1988, no centralized collection of records exists beyond district summaries; sleeping sickness case data remain in records at individual treatment centers. Data before 1988 were collected retrospectively in 2004 during visits to all treatment centers active in the 1980s. In many cases, record books were poorly stored, damaged, had lost pages, or were missing (Figure 2). Case definition was based on the primary diagnosis; all cases recorded in record books were included in this study. Cases were assigned to a year based on the patient's date of admission.

Gaps in the dataset increased before 1986. Limited data were available for the late 1970s, and no quantitative data were available for the early- to mid-1970s. Interviews were conducted with public health officials to complement and extend 1970s and 1980s data. These officials were chosen by identifying Ugandans actively involved in senior positions in sleeping sickness prevention and control from 1970 to the present and those who could be contacted. They included veterinary and public health managers at the National Ministry of Health or District Medical or Veterinary Office levels. Interviews were used to verify data for the 1980s and to classify disease magnitude for the 1970s.

Sleeping sickness magnitude was classified into 1 of 5 categories for each subcounty for each year: 1) no cases, 2) preepidemic (1–4 cases per year per subcounty), 3) low epidemic (5–15 cases per year per subcounty), 4) high epidemic (16–100 cases per year per subcounty), and 5) extreme epidemic (>100 cases per year per subcounty). These thresholds were based on anecdotal guidance from preliminary interviews and defined to facilitate

Figure 2. Records room at Bugiri Hospital sleeping sickness treatment center, Uganda.

standardized definitions of magnitude during subsequent interviews. Interviewer information was compared to available sleeping sickness records and the literature. These results were used to develop a classification database of disease magnitude by subcounty and year. Resulting data and maps were presented to informants for discussion and validation at follow-up interviews in 2005.

The boundaries of political regions changed greatly during the 34-year study period. Aggregation of subcounty data reduced the number of subcounties from 254 in 2004 to 225 for the current study period. Temporal resolution of data is consistent, and aggregation of 29 subcounties is assumed to have little effect on overall analyses.

Data Analysis

Case counts from 225 subcounties in southeastern Uganda for 34 years (1970–2003) were aggregated into 5 temporal periods for descriptive and geographic cluster analysis on the basis of epidemic progression and data availability: 1970–1975, preepidemic; 1976–1979, epidemic increase; 1980–1888, epidemic peak; 1989–1997, epidemic decrease; and 1998–2003, epidemic tail. Mean case counts per subcounty per year were calculated for periods in which case counts were available (1980–1988, 1989–1997, and 1998–2003). For earlier periods (1970–1975 and 1976–1979), only ordinal data were available. Therefore, data midpoints were calculated by using the mean of the maximum and minimum ordinal values rounded to the nearest whole number in the direction of the mode. These data were used to develop maps averaging the annual incidences of sleeping sickness for each subcounty during the interval period. Averages of annual incidence for intervals after 1980 were reclassified as sporadic (<5 cases/year), low epidemic (5–15 cases/year), or high epidemic (>15 cases/year) to match ordinal data categories for 1970s data.

To identify clusters of sleeping sickness in southeastern Uganda from 1970 to 2003, the space-time scan statistic (*14*) was used (SaTScan version 5.1 software for spatial and space-time scan statistics available from http://www.satscan.org/ [Kulldorff, Boston, MA, USA and Information Management Services Inc., Silver Springs, MD, USA]). The incidence proportions of the 225 subcounties were assumed to follow a Poisson distribution according to the underlying population size. Cluster analysis results include space-time clusters with no geographic overlap of clusters allowed and a maximum allowable cluster size of 50% of the population. Space-only and time-only clusters were excluded. Primary and secondary clusters at a significance level of $\alpha = 5\%$ are reported.

Sleeping sickness data were used in the form of case counts per year per subcounty for post-1980 records. For 1970s data, recorded values represent ordinal data (i.e., low, medium, high) rather than case counts. These were transformed to case counts by applying the ordinal minimum value to each record. Population data are based on the 1980, 1991, and 2002 population censuses for Uganda (*11*). The first 3 analysis periods (1970–1988) used 1980 census records for population counts. Analyses for the periods 1989–1997 and 1998–2003 are based on 1991 and 2002 census records, respectively.

A vector velocity map (*15*) of sleeping sickness spread was developed by using trend surface analysis (TSA) (*16,17*). TSA is a global smoothing method using polynomials in geographic coordinates, as defined by the central point of each subcounty polygon. In this case, a trend surface of the year of the first reported sleeping sickness case for each subcounty was used to explore and identify diffusion patterns and corridors of spread over time.

The year of the first recorded case was identified for subcounties in the database. Eighty-nine of 225 subcounties with no recorded cases in the 1970–2003 study period were excluded. The x- and y-coordinates of subcounty centroids were calculated from a UTM projection shapefile of southeastern Uganda using ArcMAP (ArcGIS 9, Environmental Systems Research Institute, Redlands, CA, USA). Least square regression using linear, quadratic, cubic, and higher-order polynomials of the x- and y-coordinates to predict year of first reported case was conducted in R (Foundation for Statistical Computing, Vienna, Austria, available from http://www.R-project.org). Partial differential equations (Δyear/ΔX and Δyear/ΔY) were derived from the fitted model, giving a vector of the magnitude (slope) and direction for each location. The square root of the slope equates to the velocity of diffusion.

Results

Epidemic Curve

Figure 3 shows the epidemic curve for 1970 to 2003 in southeastern Uganda, as well as a curve of the total number of subcounties infected per year. The latter curve gives an indication of the spatial extent of the disease in the region, while the former indicates the magnitude of the epidemic. The dramatic decrease in incidence in 1982 and 1983 is related to both German Red Cross intervention in 1980 in the Luuka County region (*19*) (D.B. Mbulamberi, pers. comm.) and reduced surveillance in 1982 and 1983. The number of cases and infected subcounties decreased in the 1990s. In contrast to the decrease in incidence, however, the number of infected subcounties remains well above preepidemic levels.

Incidence Maps and Cluster Detection

Figures 4–8 show maps of the average annual sleeping sickness incidence (*T. b. rhodesiense*) per subcounty in

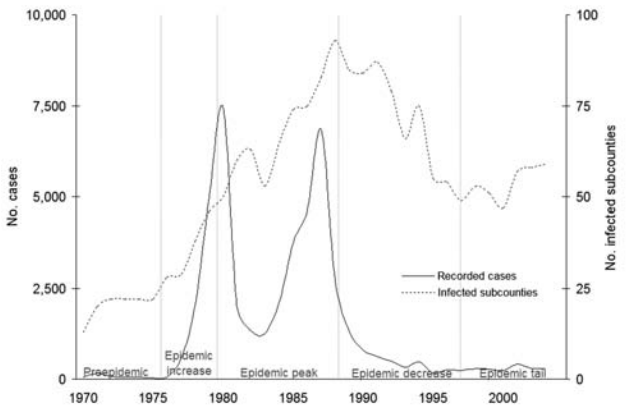

Figure 3. Number of sleeping sickness cases and infected subcounties, southeastern Uganda, 1970–2003. Number of recorded cases refer to totals for southeastern Uganda. Sources: 1970–1971, D.B. Mbulamberi, unpub. data; 1972–1975 (*18*); and 1976–2001 (Ministry of Health, 2004).

each of the 5 study periods. Legends for the 5 maps are consistent. Each map includes the location of significant (α = 5%) primary and secondary space-time clusters. Results of cluster detection analyses are discussed below for each interval period and are summarized in the Table. Dominant trends in cluster results were insensitive to maximum cluster size.

1970–1975: Preepidemic

Figure 4 shows the distribution of sleeping sickness that approximates the preepidemic zone of traditional and sporadic infection during the 1960s. Most subcounties are reported as having only a few cases per year. A significant space-time cluster was identified in the area that included the districts of Mayuge, Bugiri, and southern Iganga for 1973–1975 (Figure 4, Table). These cluster results reflect the beginning of incidence increase in these subcounties in the latter half of this period.

1976–1979: Epidemic Increase

Figure 5 shows both an increase in incidence of sleeping sickness along the Iganga/Mayuge/Jinja District borders as well as outward spread of the disease. These processes characterize the onset and increase of the sleeping sickness epidemic in 1976. A 1978–1979 space-time cluster (Figure 5, Table) of smaller size is identified northwest of the cluster for the previous period. The cluster is identified for the later years of the interval, indicating early epidemic onset and propagation, while the smaller radius of the 1978–1979 cluster reflects increased incidence at the epicenter along the Iganga border with Mayuge and Jinja.

1980–1988: Epidemic Peak

Figure 6 shows an extensive increase in both incidence and distribution of sleeping sickness that characterized this peak period of the epidemic. Detection analysis identified a cluster in 1985–1988 (Figure 6, Table) located in the same vicinity as those seen in the 2 previous intervals. The 1985–1988 cluster, in addition to the regions in the 1970s clusters, encompasses areas of Jinja, northern Iganga, and southern Kamuli Districts, indicating continued spatial spread.

1989–1997: Epidemic Decrease

Figure 7 shows the average annual incidence of sleeping sickness for the period 1989–1997. A decrease in overall incidence can be observed in conjunction with continued spatial spread. Cluster detection identified a cluster for 1989–1992 that encompassed the same areas as previous clusters, as well as the Districts of Tororo, Busia, and eastern Mukono (Figure 7, Table). In contrast to the previous periods, a space-time cluster was identified in the first years of the period. This finding reflects a shift in the epidemic from progression to regression. The larger spatial size of the cluster, however, indicates continued spread into new areas (Figure 7). Areas of increased incidence are generally shifted east.

1998–2003: Epidemic Tail

Figure 8 shows the distribution of sleeping sickness incidence for the period 1998–2003. The overall incidence of disease decreased in the southern districts, and the epidemic was characterized by pockets of disease. In addition, the disease was observed for the first time in Soroti District

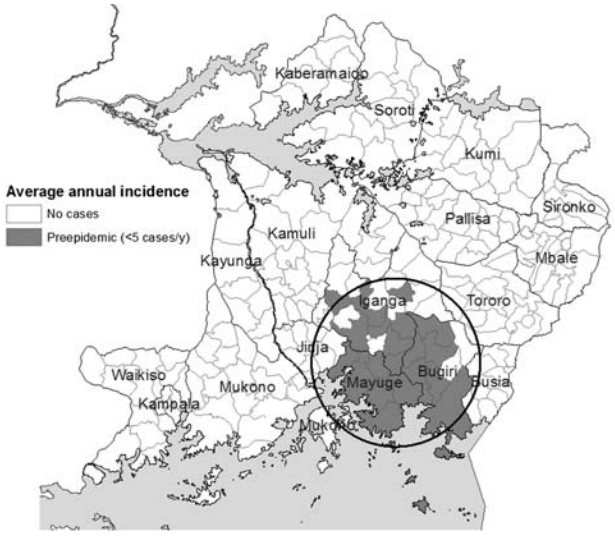

Figure 4. Sleeping sickness incidence, southeastern Uganda, 1970–1975, by subcounty. Circle indicates a significant space-time cluster at the 95% confidence level, as detected by the space-time scan test. See Table for scan test results.

Table. Cluster detection of sleeping sickness, southeastern, Uganda, 1970–2003

Interval (cluster)	Districts in most likely cluster	Cluster date	No. observed cases	No. expected cases	Relative risk*	p value	Cluster radius (km)
1970–1975	Mayuge, Bugiri, and southern Iganga	1973–1975	63	8	8.3	0.0001	41
1976–1979	Northwest shift to include northern Mayuge, Iganga, Jinja, and southeastern Kamuli	1978–1979	311	23	13.5	0.0001	29
1980–1988	Wider extent, including Mayuge, Bugiri, Iganga, Jinja, and southern Kamuli	1985–1988	13,943	1,865	7.5	0.0001	45
1989–1997	As above, plus Tororo, Busia, eastern Mukono, southern Kayunga, and southern Pallisa	1989–1992	3,176	869	3.7	0.0001	74
1998–2003 (A)†	Northwestern Iganga (Luuka county) and southern Kamuli	1999–2001	331	26	12.6	0.0001	19
(B)†	Soroti	2001–2003	263	21	12.5	0.0001	22
(C)†	Tororo (Osukulu subcounty)	2001–2002	89	7	12.9	0.0001	6
(D)†	Mukono (subcounties of Buikwe, Buyikwe, Najja, Ngogwe, and Ssi)	1998	50	4	12.5	0.0001	0‡

*Observed no. cases/expected no. cases.
†Multiple clusters were identified during 1998–2003. Letters correspond to cluster labels in Figure 8.
‡Cluster included only 1 observation representing 5 merged subcounties.

in the north of the study area (cluster B, Figure 8). Cluster detection was consistent with this distribution of outbreak pockets and foci. Four small clusters were detected (Figure 8, Table). Cluster A was detected for 1999–2001 in the subcounties along the border of Iganga and Kamuli Districts. Cluster B identified a new outbreak focus in Soroti District in 2001–2003, where cases were first recorded in 1998. This cluster reflects the increase in incidence in Soroti to the end of the study period. Although incidence in Tororo District peaked around 1990, small outbreak resurgence in Bugongi and Osukuru subcounties in 2001 and 2002 resulted in cluster detection in Tororo District (cluster C, Figure 8, Table) (*20*). A fourth, smaller, cluster was detected in the subcounties of Buikwe, Buyikwe, Najja, Ngogwe, and Ssi in 1998 (cluster D, Figure 8), which experienced a resurgence of incidence since an earlier peak in 1991.

Trend Surface Analysis

The results from trend surface analysis are summarized in a velocity vector map (Figure 9). The velocity and direction of diffusion for each coordinate location were mapped to show the movement and instantaneous rate of *T. b. rhodesiense* sleeping sickness diffusion in southeastern Uganda over the study period. TSA with high-order polynomials is sensitive to data anomalies at the edge of the study area (*15*). Less data are available at the study area boundaries; velocity vector size and direction are therefore less reliable and may not be accurate at the edge of the study area. For these reasons, 9 velocity vectors were removed from the vector diffusion map (Figure 9).

The average velocity of sleeping sickness spread over 34 years in southeastern Uganda from 1970 to 2003 was 5 km/year. Velocity of movement was highest early in the epidemic (Figure 9), when sleeping sickness spread out of its primary focus in southern Iganga District. The epidemic diffused outward in a relatively constant sphere of diffusion from this epicenter. A corridor of movement can be

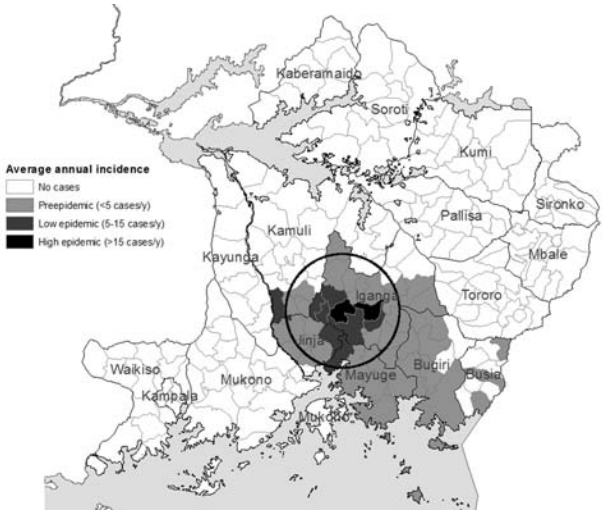

Figure 5. Sleeping sickness incidence, southeastern Uganda, 1976–1979, by subcounty. Circle indicates a significant space-time cluster at the 95% confidence level, as detected by the space-time scan test. See Table for scan test results.

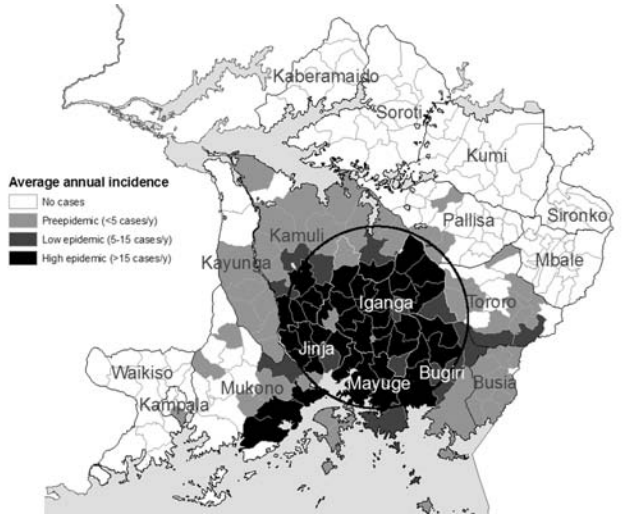

Figure 6. Sleeping sickness incidence, southeastern Uganda, 1980–1988, by subcounty. Circle indicates a significant space-time cluster at the 95% confidence level, as detected by the space-time scan test. See Table for scan test results.

observed on the eastern fringe of the study area, moving through Busia and Tororo Districts. However, this fringe area should be interpreted with caution because of potential edge effects. The disease moved distinctly north and east into Soroti District. Areas of most rapid spread appear to be the extensions of these 2 corridors north from Soroti and Tororo. These results are consistent with recent detection of cases in the districts of Kumi, Kaberamaido, and Lira (*9*,*10*) adjacent to or north of Soroti. Velocity vectors also showed spread west and east. However, Figures 4–8 suggest that much of this horizontal diffusion occurred before the 1990s.

Discussion

The reliability of data for the 1970s and 1980s is subject to detection and recording bias associated with periods of passive surveillance, missing record books, and recall bias of interviewees. The creation of additional treatment centers over the study period and differential quality of diagnostic and treatment facilities throughout the study area may contribute to spatial bias in the data. Odiit et al. (*20*–*22*) discuss the potential for misdiagnosis of cases, selective entry bias around treatment centers, and underdetection of sleeping sickness. Aggregation of cases by subcounty reduces the potential for clustering around individual treatment centers, and unless differential misdiagnosis occurs, it will not critically affect the spatial patterns seen. The data must be interpreted with caution in the context of data reliability and potential biases. Results should be considered exploratory and descriptive; data are not appropriate for direct causal inferences. The results are, however, useful for characterizing broad trends; where historical trends in processes observed are consistent with hypotheses, results can inform current and future research.

T. b. rhodesiense sleeping sickness in southeastern Uganda from 1970 to 2003 followed a pattern of radial spread from its center in southern Iganga District. From 1976 to the 1990s (Figures 4–7), the epidemic trend coincided with civil unrest and political instability in the country. The increase in the epidemic (1976–1979) occurred at a time of increasing political and economic instability, while the peak epidemic period (1980–1988) occurred during the height of political and economic collapse. The decrease in the epidemic (1989–1997) also coincides with increasing stabilization of politics and civil unrest in Uganda. The epidemic trend observed is consistent with our hypothesis (process A) that incidence increases in regions with a history of infection because of changes in human-vector exposure that push the probability of transmission above the required threshold for focal outbreaks. Uganda in the 1970s and 1980s experienced extensive internal displacement of the rural population, illegal human and cattle movements, growth of favorable tsetse habitats on cotton and coffee plantations, and collapse of sleeping sickness prevention and control activities (*8*,*19*,*23*). These events likely contributed to increased human-vector contact and sleeping sickness transmission in the districts around the preepidemic zone of infection.

After the decrease in the epidemic in the 1990s, new outbreaks have been observed in Soroti (1998, Figure 8), Kaberamaido, Kumi, and Lira (2004–2005) Districts (*7*,*9*,*10*). The introduction of the parasite into Soroti District has been linked to cattle restocking from infected

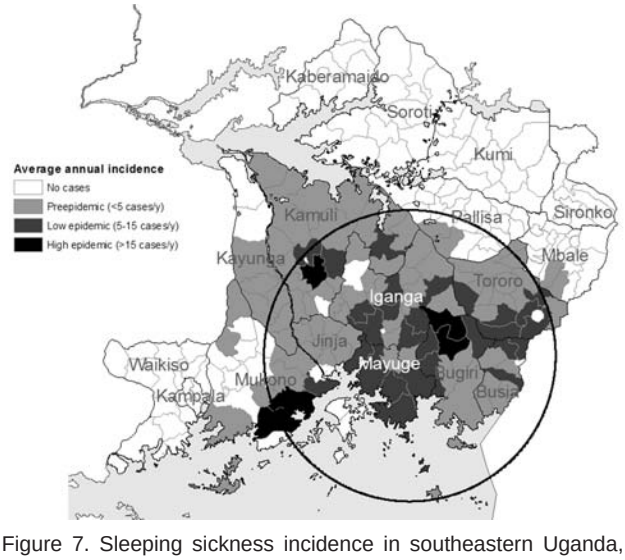

Figure 7. Sleeping sickness incidence in southeastern Uganda, 1989–1997, by subcounty. Circle indicates a significant space-time cluster at the 95% confidence level, as detected by the space-time scan test. See Table for scan test results.

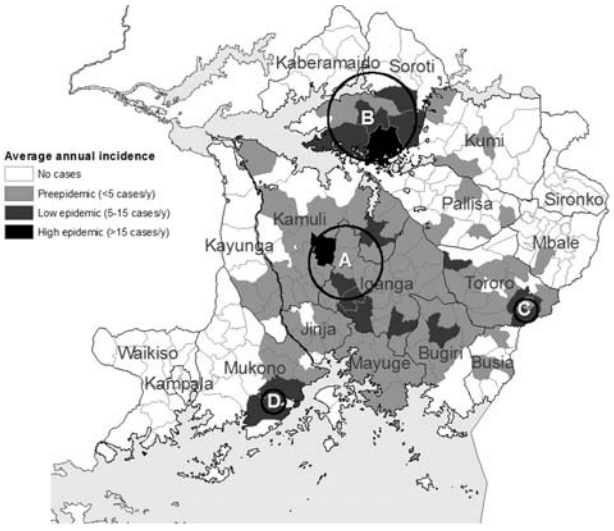

Figure 8. Sleeping sickness incidence in southeastern Uganda, 1998–2003, by subcounty. Circles indicate significant primary (A) and secondary (B, C, and D) space-time clusters at the 95% confidence level, as detected by the space-time scan test. Letters correspond to cluster results in Table. See Table for scan test results.

southern districts (*7*). Whether more recent spread into new districts is related to cattle movements is unclear. Postepidemic spread into previously uninfected and peripheral districts since the late 1990s is consistent with our hypothesized second process, which is characterized by parasite spread into new areas through movements of livestock vector. Continuing civil conflict near and within these areas is of particular concern. Once established in new regions, processes of transmission may change from introduction of parasites through cattle movements (process B) into proliferation and continued transmission through increased vector-human exposure resulting from effects of civil conflict (process A). The observed historical trends in sleeping sickness, in the context of our hypotheses, support the likelihood of continued spread of *T. b. rhodesiense* north from newly infected regions in central Uganda.

Figure 9. Velocity vectors (arrows) for the spread of sleeping sickness between subcounties in Uganda. Arrow length is proportional to velocity of spread.

Figure 3 suggests that while the number of recorded cases remains low, those cases are coming from an increasing large area. Decreased sleeping sickness surveillance systems in Uganda (D.B. Mbulamberi, pers. comm.) may be missing undetected increases in cases while still detecting infection at the subcounty level. The likelihood of such detection bias is unclear, although a similar difference between recorded cases and recorded subcounties infected preceded the epidemic increase in 1976 (Figure 3). Sleeping sickness is a highly focal disease often characterized by distinct outbreaks in a specific area or village. This outbreak pattern has been smoothed by aggregation of cases to the subcounty level. In spite of the highly focal nature of sleeping sickness, the results suggest a pattern of observable, continuous, and potentially predictable spread of *T. b. rhodesiense* sleeping sickness in Uganda when data are smoothed to the subcounty level.

The description and characterization of historical reemergence of sleeping sickness in southeastern Uganda can be used to guide and complement research into the causal processes determining the observed patterns of incidence and spread. These patterns are consistent with our hypotheses of 2 dominant processes of sleeping sickness transmission in southeastern Uganda. First, in regions where disease currently occurs or has recently occurred, localized outbreaks are triggered by changes in vector-human exposure or vector numbers, which push the probability of transmission above threshold levels. This process was observed around the traditional infection zone in southeastern Uganda during the 1976–1990 epidemic. Second, in regions where disease has not recently occurred, spread is facilitated by transmission of the parasite thorough livestock. This is currently being observed in the spread of infection to districts in central Uganda that were not infected during the previous epidemic.

Conclusions support further research and intervention related to parasite transmission through cattle movements and potential changes in vector-human exposure in central Ugandan districts. Such analyses are particularly relevant in the context of continued spread of *T. b. rhodesiense* sleeping sickness in Uganda, potential merging with *T. b. gambiense* subspecies in northwest regions (*24*), and ongoing civil unrest in north-central regions.

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Mycobacterium intermedium Granulomatous Dermatitis from Hot Tub Exposure

Randall S. Edson,* Christine L. Terrell,* W. Mark Brutinel,* and Nancy L. Wengenack*

Nontuberculous mycobacteria, which are widespread in the environment, frequently cause opportunistic infections in immunocompromised patients. We report the first case of a patient with chronic granulomatous dermatitis caused by a rarely described organism, *Mycobacterium intermedium*. The infection was associated with exposure in a home hot tub.

Nontuberculous mycobacteria are major causes of opportunistic infection in immunocompromised patients. These organisms are widespread throughout the environment, including water and soil (*1*,*2*). We report the first case of a patient with chronic granulomatous dermatitis caused by a rarely described organism, *Mycobacterium intermedium*, which was associated with exposure in a home hot tub.

The Study

A 55-year-old nonimmunosuppressed man first sought medical attention in March 2000 for an indurated papular rash on his back (Figure, panels A and B). A biopsy showed granulomatous dermatitis, but all cultures and stains, including those for fungi and mycobacteria, were negative. In September 2000, another biopsy showed histologic results identical with those of the previous biopsy. Mycobacterial cultures at this time were positive for *M. intermedium*; tissue stains were negative.

To confirm the microbiologic result, another biopsy was performed in October 2000, at which time a single auramine-rhodamine–positive staining result was noted in the tissue. Culture at this time was negative for mycobacteria. He was treated with topical corticosteroids with partial improvement. In March 2001, *M. intermedium* was recovered from 2 separate biopsy specimens. On the basis of susceptibility data, treatment with isoniazid, ethambutol, and clarithromycin was initiated.

In July 2001, considerable improvement was noted. That same month, the patient received methylprednisolone (1 g intravenously) for 5 days for an ill-defined neurologic condition. At a follow-up visit in June 2002, he reported a 2-week history of diminished vision in the left eye. Ethambutol, isoniazid, and clarithromycin were withdrawn. At that time, his dermatitis was somewhat improved but not entirely resolved. In October 2002, he came to the clinic with new lesions on his back. During this visit, he reported immersion twice a day in a home hot tub, which provided temporary relief for his chronic back pain. Additionally, he reported that when sitting in the tub, his upper back was in contact with several nozzles that delivered water under high pressure. He was advised to refrain from using the hot tub.

Three months later, he had almost complete resolution of the skin lesions with no further medical treatment (Figure, panel C). At that time, a water sample obtained from the patient's hot tub was positive for *M. intermedium*.

Skin lesion biopsy specimens were placed in sterile beef nutrient broth and cultured in a mycobacteria growth indicator tube (MGIT, Becton Dickinson, Sparks, MD, USA) supplemented with oleic acid, albumin, dextrose, catalase growth supplement (OADC, Becton Dickinson), and an antimicrobial drug mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin [PANTA, Becton Dickinson]. The MGIT was incubated on the BACTEC MGIT 960 instrument (Becton Dickinson) at 37°C, and growth was shown by an increase in fluorescence after 9 days. A cytospin slide prepared from the broth was positive for acid-fast bacilli by Kinyoun stain. The MGIT broth was subcultured to a Middlebrook 7H10/S7H11 agar biplate and incubated at 37°C in 5% CO_2 to obtain a pure culture of the organism for subsequent identification.

To obtain a culture of hot tub water, the patient was instructed to fill the hot tub with water and allow it to stand for 2 weeks before sampling. A sample of water was collected from the hot tub in sterile, screw-top tubes. The water was concentrated by centrifugation and treated with N-acetyl-L-cysteine and 1% sodium hydroxide to remove bacteria that might overgrow more slowly growing mycobacteria. The specimen was then injected into an MGIT for incubation at 37°C. The MGIT showed fluorescence (bacterial growth) after 1 day of incubation, and the Kinyoun stain was positive for acid-fast bacilli. The MGIT broth was subcultured to a Middlebrook 7H10/S7H11 biplate and incubated at 37°C in 5% CO_2 to obtain a pure culture of the acid-fast bacilli for identification.

The skin lesion and hot tub isolates were tested by using nucleic acid hybridization probes (AccuProbe, Gen-Probe Inc, San Diego, CA, USA) to rule out *M. tuberculosis* complex, *M. avium* complex, and *M. gordonae*. Polymerase chain reaction was performed to amplify mycobacterial DNA, and the amplified DNA was

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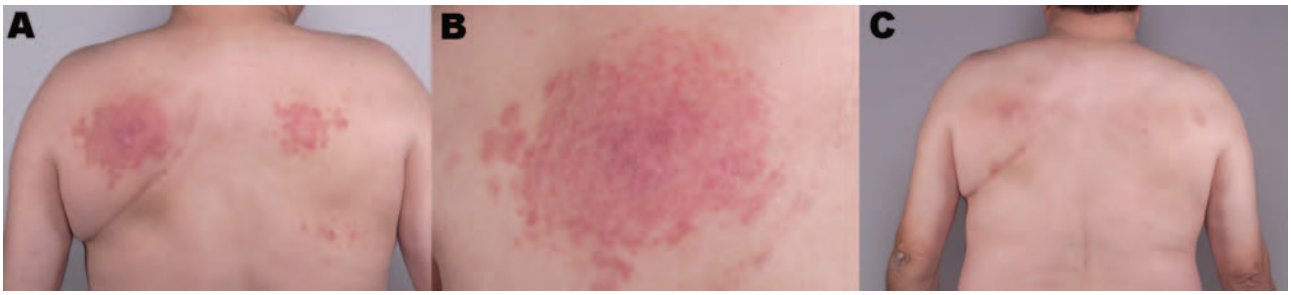

Figure. A) Appearance of rash on the patient's back at initial treatment. B) Close-up of the rash shown in panel A. C) Patient's back showing near resolution of rash after discontinuing use of the hot tub.

sequenced by using 16S rDNA sequencing as previously described (*3*). By using a distance score of ≤1% from the sequencing library entry to identify the species, the isolate obtained from the skin lesions and the hot tub water was identified as *M. intermedium*.

Conclusions

This is the first clearly documented case of granulomatous dermatitis caused by *M. intermedium*, a novel, slow-growing mycobacterium originally isolated from the sputum of a patient with pulmonary disease (*4*) and recently described in an elderly man (*5*). *M. intermedium* isolated from our patient's hot tub was responsible for a chronic granulomatous dermatitis, which appeared to be refractory to appropriate antimicrobial therapy because of repeated exposure to contaminated water. The nodular eruption resolved only when use of the hot tub was discontinued. The distribution of the skin lesions on his back corresponded to the position of the high-pressure water jets.

Mycobacteria are commonly recovered from various environmental and potable water sources. Covert et al. (*1*) isolated nontuberculous mycobacteria from 38% of sampled drinking water, and Collins et al. (*2*) found a wide variety of mycobacterial species (*M. kansasii*, *M. xenopi*, *M. avium*, *M. marinum*, *M. fortuitum*, *M. chelonei*, *M. gordonae*) from both domestic and environmental water sources. These organisms are inherently resistant to disinfectants such as chlorine, which contributes to their persistence, even in treated water (*6*).

Public and private hot tubs, whirlpools, and public spas are increasingly popular in the United States. Public spas are periodically inspected to ensure that minimum hygienic standards for water safety are maintained. A recent report, summarizing the results of several such inspections, suggests widespread violations (*7*); >50% of these inspections indicated significant deficiencies in disinfection, pH control, and general maintenance. Private facilities such as home hot tubs and whirlpools are not subject to any surveillance or quality control.

Several clinical syndromes have been attributed to waterborne mycobacteria. Several investigators (*8–11*) have reported an association between hypersensitivity pneumonitis and spa-associated contamination with *M. avium* complex. Aubuchon et al. (*12*) described a patient with an amputation stump infection caused by *M. fortuitum* acquired from a home hot tub, and Lee et al. (*13*) reported a 24-year-old woman who acquired a soft tissue infection caused by *M. abscessus* from a public bath where she was employed. A recent report (*14*) described an outbreak of lower extremity furunculosis caused by *M. fortuitum* that affected >115 patrons of a nail salon; culture of the water from the whirlpool foot bath showed contamination with *M. fortuitum*.

Our case report highlights the paramount importance of medical history in the care of patients with enigmatic illnesses. The patient's rash failed to respond to seemingly appropriate therapy over a 2-year period because of constant reexposure to the contaminated water. Had a familial outbreak occurred, the diagnosis may have been more obvious. In this case, the patient was the only person using the hot tub, and a point source was not suspected. Only with repeated questioning was an association with the hot tub established. Clinicians should consider asking patients about hot tub, whirlpool, and spa exposure in the appropriate clinical context, such as cutaneous disease or pulmonary infiltrates for which no clear explanation exists.

Dr Edson is a consultant at the Mayo Clinic and professor of medicine at the Mayo Clinic College of Medicine, Rochester, Minnesota. His research interests include residency education and unusual manifestations of infectious diseases.

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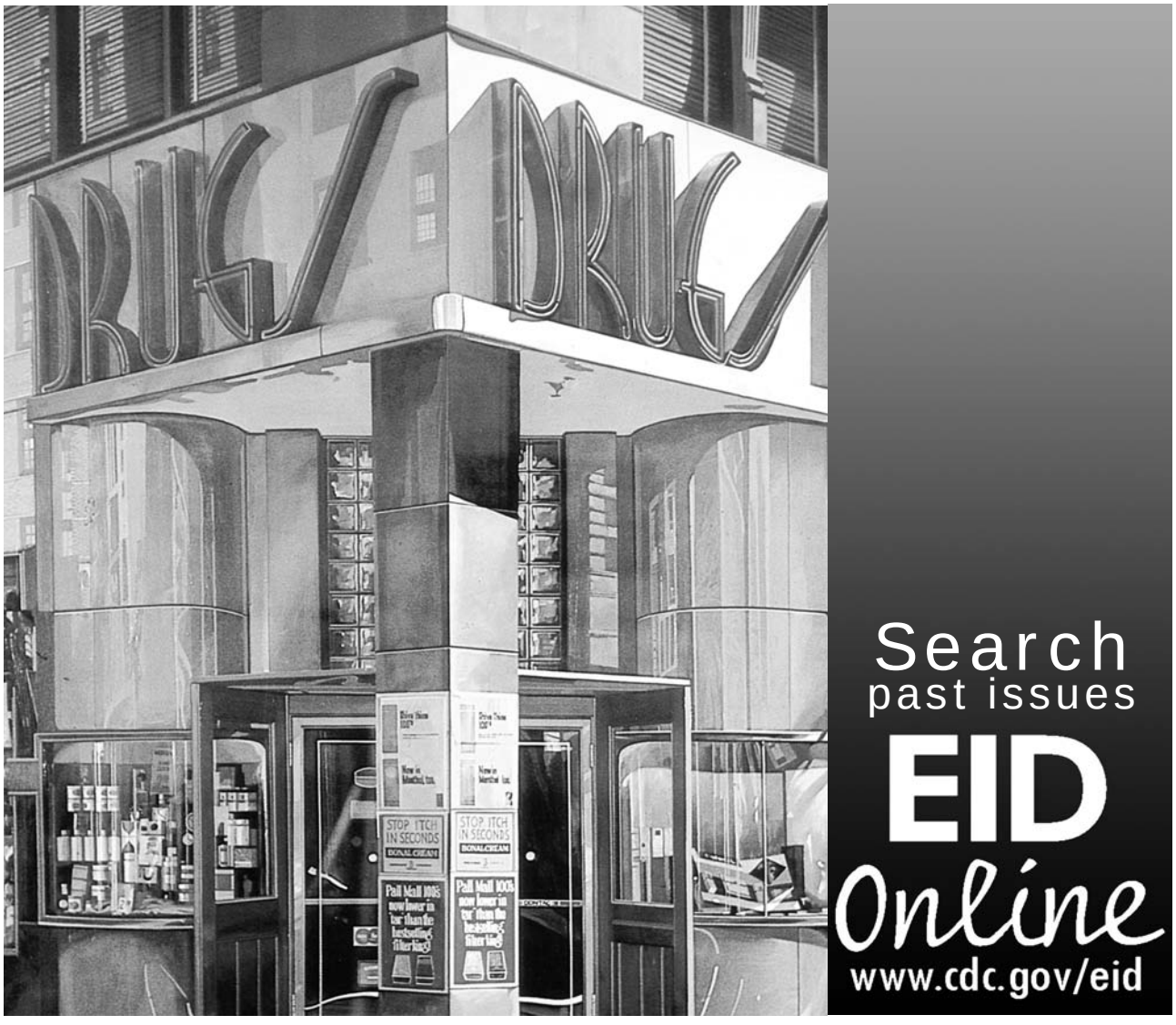

Molecular Characterization of Rotavirus Gastroenteritis Strains, Iraqi Kurdistan

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Of 260 children with acute diarrhea in Erbil, Iraqi Kurdistan, 96 (37%) were infected with rotavirus. Reverse transcription–polymerase chain reaction identified G1, G4, G2, G9, P[8], P[6], and P[4] as the most common genotypes. Eight G/P combinations were found, but P[8]G1 and P[4]G2 accounted for >50% of the strains.

Rotavirus is the single most important cause of severe gastroenteritis in young children throughout the world. Globally, an estimated 702,000 children die each year due to rotavirus diarrhea (*1*). This large impact of rotavirus disease has speeded the development of rotavirus vaccines, and 2 live, attenuated rotavirus vaccines are expected to be available for global use within the next few years (*1*). Therefore, determining the prevalence and types of rotaviruses within regions is essential to prepare for introducing a vaccine.

Rotavirus, a member of the family *Reoviridae*, has a triple-layered capsid that contains 11 segments of double-stranded genomic RNA. While protective immunity against rotavirus infection is not completely understood, serotype-specific immunity is believed to play a major role (*1*). Rotavirus serotypes are defined by genome segment 4 for the P (protease-sensitive protein) type and by genome segment 9 (or 7 or 8, depending on the strain) for the G (glycoprotein) type. Fourteen G types exist, of which G1–G4 are commonly found in children with diarrhea, but a recent increase in the detection of serotype G8 and G9 strains has captured considerable attention (*2–4*). While >24 P types have been reported in the literature, only P[4], P[6], and P[8] are commonly found among human rotaviruses (*1–3*).

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In Iraq, the death rate in children <5 years of age was reported to be 130/1,000 for boys and 120/1,000 for girls in 2003 (*5*). Diarrhea is a major cause of illness and death in Iraqi children; however, little information exists about the origin of childhood diarrhea. Only a single study showed that rotavirus accounted for 24% of acute diarrhea in hospitalized children in Basrah (*6*).

The Study

This descriptive, cross-sectional study of 6 weeks' duration was undertaken at Erbil Paediatric Hospital in Iraqi Kurdistan between March and May 2005. The study recruited 260 children from 1 month to 5 years of age who were admitted with acute diarrhea (defined as the passage of watery or loose stools ≥3 times per day for <2 weeks' duration). Basic demographic, epidemiologic, and clinical information were collected prospectively, according to a pro forma. Ethical approval for the research was obtained from the review boards of the Liverpool School of Tropical Medicine and Erbil Paediatric Hospital. The hospital serves a population of ≈1.5 million, and ≈3,116 births per month occur in this population.

A commercial enzyme-linked immunosorbent assay (ELISA) was used to detect rotavirus antigen (Rotaclone, Meridian Diagnostics, Cincinnati, OH, USA). Stool samples were then stored frozen in the laboratory of the study hospital until they were transported to Liverpool for rotavirus genotyping and electropherotyping. All samples (66) with an absorbance equal to or greater than the positive control for the ELISA were subjected to genotyping. Rotavirus genomic RNA was extracted with guanidine isothiocyanate, followed by adsorption to and elution from silica particles according to the method described by Gentsch et al. (*7*). The purified RNA was then used to determine the P type and G type of rotavirus present in the stool specimens by reverse transcription-polymerase chain reaction as described by Gentsch et al. (*7*) and by Gouvea et al. (*8*). Rotavirus electropherotypes were determined by polyacrylamide gel electrophoresis according to the method described by Koshimura et al. (*9*), with some modifications.

Of 260 stool specimens tested by ELISA, 96 (37%) were positive for rotavirus. Rotavirus-positive patients had a mean age (SD) of 9.3 (8.5) months compared to 11.1 (10.1) months in the rotavirus-negative patients. These results suggest that rotavirus positive cases were slightly younger, although the difference was not statistically significant ($p = 0.14$). Rotavirus-positive patients were similar to rotavirus-negative patients in most of the epidemiologic and clinical characteristics (data not shown). However, rotavirus-positive patients were more likely to exhibit vomiting and have a shorter duration of diarrhea ($p<0.01$ for both analyses).

Of the 66 rotavirus strains that underwent molecular characterization, 25 (38%) were G1, 11 (17%) were G2, 13 (20%) were G4, and 7 (11%) were G9. Four (6%) were mixed infections (3 G1/G2, 1 G2/G4), and 6 (9%) were G nontypeable. A total of 7 (11%) were P[4], 10 (15%) were P[6], and 45 (68%) were P[8]. One showed mixed P[4] and P[8] genotypes (mixed with G1/G2), and 3 (5%) were P nontypeable. None of the rotaviruses was both G and P nontypeable.

A total of 8 different P and G genotype combinations were detected (Table). The most common combinations were P[8]G1 (19, 33%), P[8]G4 (12, 21%), P[4]G2 (6, 11%), P[6]G1 (6, 11%), and P[8]G9 (6, 11%). The unusual combination of P[6]G9 was detected in 1 of the patients.

An electropherotype was obtained for 50 of the 66 genotyped strains. Of these, 11 (22%) had a short electropherotype, and 39 (78%) had a long electropherotype (Table). Most of the short electropherotypes were the expected G2 strains; however, 1 strain (P[8]G9) also had a short electropherotype.

Table. Rotavirus genotypes and electropherotypes*

Genotype	No.(%) fully typeable strains	Electropherotype†
P[4]G2	8 (15)	Short (7/8)
P[6]G1	6 (11)	Long (5/6)
P[6]G4	1 (2)	ND
P[6]G9	1 (2)	Long
P[8]G1	19 (33)	Long (13/19)
P[8]G4	12 (21)	Long (12/12)
P[8]G9	6 (11)	Long (4/6); short (1/6)
P[6]GNT	2	Long (2/2)
P[8]GNT	4	Long (2/4)
P[NT]G2	3	Short (3/3)

*Four rotavirus infections were mixed: P[8]G1/G2 (2), P[4]G2/G4 and P[4]/[8]G1/G2. †Indicates number of strains electropherotypeable in the genotype combination; ND, not determined.

Conclusions

The only other study of viral gastroenteritis from Iraq (Basrah in the south) demonstrated that 24% of children with acute gastroenteritis were infected with rotavirus (*6*). This figure is somewhat lower than the 37% detection rate in our study. Moreover, the prevalence we found is similar to those reported from neighboring countries such as Iran (35%) (*10*), Jordan (33%) (*11*), Kuwait (40%) (*12*), and Turkey (37%) (*13*). However, our study was undertaken over a 6-week period from the end of March to the beginning of May 2005. No information is available on the seasonal prevalence of rotavirus infection in Iraq, and a longer study is warranted to determine the true prevalence of rotavirus infection and its seasonality in northern Iraq. However, the peaks of rotavirus infection in Iran, Kuwait, and Turkey were February–March, March–May, and December, respectively (*10*,*12*,*13*). More than 75% of our cases of rotavirus diarrhea occurred in children <1 year of age, with an overall mean age of slightly more than 9 months. This pattern is similar to that in many developing countries. In Jordan the mean age of children with rotavirus diarrhea was 7.2 months (*10*). However, in other countries in the region the distribution was different; 30% of the infants with rotavirus in Iran were <1 year of age (*10*,*12*), 50% in Kuwait were <1 year of age, and 63% in Turkey were <2 years of age (*13*).

Although this study period was brief, we detected a variety of rotavirus strains. Four of the major global human rotavirus genotypes (G1, G2, G4, G9) were detected, as were each of the major P genotypes (P[4], P[6], P[8]). In Iran, in a study undertaken in 2001 and 2002, only G1 and G2 rotaviruses were detected, and the only P types were P[4] and P[8] (*10*), and in Turkey over a 2-year period (2000–2002), G types G1–G4 and G9, as well as each of the 3 major human P types were found (*14*). In Iraq, the combinations P[8]G1 and P[8]G4 accounted for >50% of the strains of rotavirus. In Iran, P[8]G1 accounted for 95% of the strains, but P[8]G4 was not detected (*10*). In Turkey, P[8]G4 (42%) and P[8]G1 (27%) accounted for more than two thirds of the strains (*14*). G3 rotaviruses were not detected in Iraq or Iran, and in Turkey only 1 of the 65 strains was of genotype G3. Genotype G9 was detected in 13% of the Iraqi strains, a similar finding to results in Turkey (*14*). We also detected mixed rotavirus infections in 6% of our patients, again similar to the findings in Turkey (*14*). The presence of mixed rotavirus infections indicates that new rotavirus strains may evolve by reassortment (*1*–*3*).

Finally, among the G9 strains, one P[6]G9 had a long electropherotype, and one P[8]G9 had a short electropherotype. The P[6]G9 and P[8]G9 strains were both cultured and subgrouped by ELISA with monoclonal antibodies and found to be of subgroup II. Partial sequences (831 bp) were obtained for their VP7 genes (AB247941 and AB247943; available from the DNA Data Bank of Japan: www.ddbj.nig.ac.jp). They showed 99.4% similarity to each other and >99% similarity to strains from Australia (AY307087), Belgium (AY487858, AY487856), and India (RG9491165). A strain similar to our P[6]G9, called variant 3, was first detected in India, and strains similar to our P[8]G9, called variant 2, have been described in Bangladesh and in the United States (*15*).

Although the major global genotypes (except for G3 strains) were detected, clearly, rotavirus strains are continuing to diversify in Iraq and other parts of the region. This circumstance may pose challenges to the efficacy of rotavirus vaccines.

Acknowledgments

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Dr Ahmed is a pediatrician who conducted this research as part of the requirements for his master's degree in tropical pediatrics. His research interests are in viral gastroenteritis and respiratory tract infections.

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Clostridium difficile Ribotype 027, Toxinotype III, the Netherlands

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Outbreaks due to *Clostridium difficile* polymerase chain reaction (PCR) ribotype 027, toxinotype III, were detected in 7 hospitals in the Netherlands from April 2005 to February 2006. One hospital experienced at the same time a second outbreak due to a toxin A–negative *C. difficile* PCR ribotype 017 toxinotype VIII strain. The outbreaks are difficult to control.

Since March 2003, outbreaks of severe cases of *Clostridium difficile*–associated disease (CDAD) were reported in hospitals in Montreal and Quebec (*1*,*2*). Increased virulence was suspected, since the proportion of patients with CDAD who died within 30 days after diagnosis rose from 4.7% in 1991–1992 to 13.8% in 2003 (*1*). In addition, the Centers for Disease Control and Prevention reported a growing threat of CDAD in US hospitals and found the strain to be associated with high illness and death rates during hospital outbreaks in 11 states (*3*). The increased virulence was considered to be associated with the production of a binary toxin and an increased production of toxins A and B (*4*). Further characterization of this strain showed that it belonged to toxinotype III, pulsed-field gel electrophoresis (PFGE) type NAP1, restriction endonuclease analysis group BI, and polymerase chain reaction (PCR) ribotype 027 (*2*,*3*). Toxinotyping involves detecting polymorphisms in the toxin A and B and surrounding regulatory genes, an area of the genome known collectively as the pathogenicity locus or PaLoc (*5*). By toxinotyping, 24 different types can be recognized, whereas the library of PCR ribotypes comprises 116 distinct types of *C. difficile* identified on the basis of differences in amplification profiles generated (*6*). The PCR ribotype 027, toxinotype III, strain is resistant to ciprofloxacin and the newer generation of fluoroquinolones, such as gatifloxacin, levofloxacin, and moxifloxacin (*3*). Exposure of patients to fluoroquinolones and cephalosporins is recognized as a risk factor for CDAD caused by 027 (*2*,*3*). Increasing use of fluoroquinolones in US healthcare facilities may have provided a selective advantage for this epidemic strain and promoted its widespread emergence.

The Outbreaks

In July 2005, the medical microbiologic laboratory at the Leiden University Medical Center was requested to type *C. difficile* strains from an outbreak in a hospital (hospital l) in Harderwijk (Figure, Table). The incidence of CDAD in the hospital had increased from 4 per 10,000 patient admissions in 2004 to 83 per 10,000 admissions from April through July 2005. Cultured isolates were subsequently identified as toxinotype III and PCR ribotype 027 (*7*). The strain also had the binary toxin genes and contained an 18-bp deletion in a toxin regulator gene (*tcdC*). As determined by E test (AB Biodisk, Solna Sweden), the isolates were resistant to erythromycin (MIC >256 mg/L) and ciprofloxacin (MIC >32 mg/L) and susceptible to clindamycin (MIC 2 mg/L) and metronidazole (MIC 0.19 mg/mL). Measures taken by the hospital included isolating all patients with diarrhea until 2 tests were negative for *C. difficile* toxin, cohorting all *C. difficile*–infected patients on a separate ward, banning all fluoroquinolone use, and limiting use of cephalosporins and clindamycin. A

Figure. Location of the hospitals with outbreaks of *Clostridium difficile*–associated diarrhea in the Netherlands. The numbers correspond with those in the Table.

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Table. Characteristics of 9 hospitals with patients with *Clostridium difficile*–associated diarrhea due to PCR ribotype 027, toxinotype III*

Hospital no. and setting	No. beds	Admissions: Incidence/ 10,000, before outbreak	Admissions: Maximum incidence/ mo/10,000, during outbreak†	Date of outbreak onset	Total no. CDAD patients in given period, 2005	Deaths, 30 d	No. strains studied	No. toxinotype III, PCR ribotype 027 strains
1. Harderwijk	341	4	83	Apr 2005	51, Apr–Nov	3	30	19
2. Amersfoort	600	11	87	May 2005	85, Jan–Dec	19	50	15
3. Utrecht	1,013	16	–	No outbreak	37, Jun–Dec	Unk.	17	6
4. Nieuwegein	584	11	–	No outbreak	13, Jan–Dec	Unk.	4	1
5. Amsterdam	1,002	38	52	June 2005	68, Jan–Oct	1	28	12
6. Amsterdam	310	10	66	Apr–May 2005	42, Jan–Oct	Unk.	34	16
7. Haarlem	744	7	27	2004	66, Jan–Dec	Unk.	9	7
8. Hoofddorp	455	3	76	Jan 2005	73, Jan–Dec	Unk.	8	8
9. Beverwijk	383	4	47	2002	24, Jan–Dec	Unk.	4	3

*PCR, polymerase chain reaction; CDAD, *Clostridium difficile*–associated diarrhea; unk., unknown.
†Timeframe 2–4 mo.

case-control study is being performed in the hospital to determine risk factors for acquiring this strain, and a follow-up study will determine the rate of complications and relapses. As of January 2006, the situation appears to be under control since the number of patients per month with positive test results has decreased. All 9 CDAD cases from September 2005 to January 2006 were caused by non-027 ribotypes. Therefore, cohort isolation and the limitation on antimicrobial agents have been stopped.

A second epidemic occurred in another hospital 30 km from the first hospital (hospital 2, Amersfoort) and was probably related to the outbreak in hospital 1 through a transferred patient with CDAD. Isolates obtained from patients were indistinguishable from the Harderwijk isolates. After the index patient was transferred, the incidence of CDAD, which had been 2–3 cases per month for the last 2 years, rose to an average of 15 cases per month during May, June, and July. From August to December, the number of CDAD patients per month was 7, 7, 8, 14, and 10, respectively. Of the 85 CDAD patients found through December 2005, 19 (22%) patients died, and 16 (19%) had relapses. Of 50 strains characterized at the reference laboratory, 15 belonged to PCR ribotype 027, and 14 belonged to PCR ribotype 017, toxinotype VIII. The 017 strain had a deletion of the toxin A gene, did not contain genes for binary toxin production, and had a normal *tcdC* gene.

In response to the outbreaks in the Netherlands, the Centre for Infectious Disease Control at the National Institute for Public Health and the Environment in Bilthoven organized a meeting with experts in the fields of microbiology, infectious diseases, infection control, and epidemiology. The team agreed to combine parts of existing national hospital guidelines relevant for infection control of CDAD and to use national and international experience in drawing up specific CDAD guidelines for infection control and treatment separate for hospitals and nursing homes. Diagnostic facilities were increased and made accessible for all microbiology laboratories in the Netherlands. Relevant professionals were informed through different communication channels, including various scientific societies (*7*). Plans were made to register and monitor new outbreaks. Laboratories were encouraged to send patient isolates or fecal samples for typing to the reference laboratory in Leiden when an outbreak was suspected on the basis of an increase in monthly incidence or a rapid spread of clinically suspected cases.

Subsequently, 3 hospitals in the western part of the country (hospitals 7–9) also reported an increase in incidence of severe CDAD. In 2005, the public health laboratory serving these 3 hospitals diagnosed CDAD in 163 patients. Of 21 strains sent to the reference laboratory, 18 were identified as PCR ribotype 027, toxinotype III (Table). Retrospectively, an increase of CDAD was first evident in July 2004 for hospital 7 and in 2002 for hospital 9. The public health laboratory diagnosed CDAD in 120 patients in 2004, in 58 in 2003, and in 47 in 2002. No strains or fecal samples before 2005 were available for typing. A nursing home in the same region was also found to have patients with CDAD due to PCR ribotype 027, with evidence of spread within the facility. No epidemiologic relationship could be established between this region ad that of the first 2 outbreaks.

Two hospitals in the center of the Netherlands (hospitals 3 and 4) did not notice an increase in the incidence of patients with CDAD but submitted strains to the reference laboratory for typing. Type 027 was found in 6 (35%) of 17 and 1 (25%) of 4 isolates tested, respectively. None of the patients with CDAD due to type 027 had severe disease.

A cluster of 12 patients with CDAD by PCR ribotype 027, toxinotype III, was reported in July and August in a large teaching hospital in Amsterdam (hospital 5). One patient died from consequences of CDAD, and severe complications developed in 2 other patients. Another hospital in Amsterdam (hospital 6) also reported an increase of

severe cases of CDAD in July 2005 in geriatric patients. Strains cultured from fecal samples of 7 patients in August 2005 showed PCR ribotype 027, toxinotype III.

Conclusions

Shortly after the reports in June 2005 of the detection of *C. difficile* PCR ribotype 027, toxinotype III, in English hospitals, this more virulent type was detected in the Netherlands (*7*,*8*). More recently, the reference laboratory at Leiden University Medical Center also detected this strain in samples from Belgium as a causative agent of outbreaks of CDAD (*9*). The virulence factors of this emerging strain are not well understood. It contains a binary toxin, but the importance of binary toxin as a virulence factor in *C. difficile* has not been established. The binary toxin, an actin-specific adenosine diphosphate–ribosyltransferase, is encoded by the *cdtA* gene (the enzymatic component) and the *cdtB* gene (the binding component), which are not located within the pathogenicity locus (*10*,*11*). Nonpathogenic strains that contain *cdtA* and *cdtB* genes but lack the pathogenicity locus are also capable of producing binary toxin. The binary toxin is present in ≈6% of all *C. difficile* isolates, irrespective of the toxinotype (*10*,*11*). We therefore consider it likely that the binary toxin in PCR ribotype 027, toxinotype III, strains merely reflects clonal spread of a restricted number of strains.

The importance of the 18-bp deletion in *tcdC* of the PCR ribotype 027, toxinotype III, strains is also unknown. *tcdC* is considered a negative regulator of the production of toxins A and B, but whether this 18-bp deletion results in a nonfunctional product is unknown (*3*). A recent report, however, indicates that toxinotype III isolates produce toxins A and B in considerably greater quantities in vitro than toxinotype 0 isolates (*4*). On the other hand, deletions in *tcdC* are frequently present in toxinogenic isolates. Of 32 toxinogenic strains studied in 2002, 8 belonged to toxinotypes 0, V, and VI and contained deletions in *tcdC* of 18 bp or 39 bp, although this deletion was not associated with severity of disease (*12*).

The PCR ribotype 027, toxinotype III, strain has a characteristic antimicrobial susceptibility pattern, since it is resistant to the newer fluoroquinolones and erythromycin but susceptible to clindamycin. Macrolide, lincosamide, and streptogramin B (MLSB) resistance is usually due to an *erm(B)* gene, but PCR ribotype 027 and toxinotype III strain did not contain an *erm(B)* gene. All current PCR ribotype 027 and toxinotype III strains but no historical isolates (obtained before 2001) were resistant to gatifloxacin and moxifloxacin (*3*). The resistance for ciprofloxacin and newer fluoroquinolones is not specific for the new virulent strains, since it has also been found in other common PCR ribotypes in the United Kingdom (*13*).

The observation that outbreaks due to different strains can occur simultaneously emphasizes that microbiologic monitoring is important for epidemiologic studies of CDAD. PCR ribotype 017 strain lacks a part of the toxin A gene and was first recognized as a cause of an outbreak in Canada in 1999 (*14*). Subsequently, toxin A–negative, toxin B–positive strains caused outbreaks of CDAD in Ireland (D. Drudy, pers. comm.), Argentina (M.C. Legaria, et al., unpub. data), and the Netherlands (*15*).

The outbreaks in the Netherlands are difficult to control. In the Harderwijk epidemic, using rapid diagnostic tests for CDAD and cohort isolation in combination with restricting use of fluoroquinolones and cephalosporins appeared to be successful. Outbreaks in the other hospitals are still not completely under control.

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Costs of Surgical Site Infections That Appear after Hospital Discharge

Nicholas Graves,*† Kate Halton,*† Merrilyn Curtis,* Shane Doidge,* David Lairson,‡ Marylou McLaws,§ and Michael Whitby*

Data were collected from surgical patients in the hospital and on 4 occasions postdischarge. The incidence of postdischarge surgical site infection was 8.46%. Strong evidence showed that these infections caused minor additional costs, which contradicts existing literature. We discuss why previous studies might have overstated costs.

Most cases of healthcare-acquired surgical site infections (SSI) appear after discharge from hospital (*1*); rates of postdischarge SSI between 2% and 14% have been reported (*2*). Little is known of the costs of postdischarge SSI, but 2 studies suggest that they are large (*3*–*5*) with health services and patients incurring costs and subsequent production losses. The combination of high frequency and high cost suggests that programs that reduce the risks of postdischarge SSI should be adopted, but decision makers should assess the cost-effectiveness of additional prevention efforts. This exercise requires valid estimates of the change in costs and benefits from additional prevention programs (*6*,*7*). Understanding the costs of postdischarge SSI is therefore essential. The work completed so far is valuable but demonstrates some methodologic weaknesses. Plowman et al. (*3*,*4*) assessed only patient-reported signs and symptoms of postdischarge SSI, and Perencevich et al. (*5*) relied on routine healthcare records for diagnosis/surveillance and matched case patients with controls on only 3 confounding variables.

Our study assessed the costs of postdischarge SSI. We adopted a societal perspective and included the costs incurred by healthcare services, private costs, and production losses. The research method was chosen to address the suggested weaknesses of the studies of Plowman et al. (*3*,*4*) and Perencevich et al. (*5*).

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The Study

We recruited, in consecutive order, adults (>18 years of age) admitted to 3 Australian hospitals in 2004 for knee or hip prostheses, cardiovascular procedures, femoropopliteal bypass grafts, or abdominal procedures, including abdominal hysterectomies and lower segment caesarean sections. Four infection-control research nurses recruited participants and collected data during the hospital admission process and on 4 separate occasions after surgery by visiting the patients in their homes (data collection is illustrated in the Figure). Monetary estimates of all costs were made by multiplying frequency with a cost vector for the item of service (*9*–*12*). Production losses were estimated by comparing the presurgery level of (unwaged and waged) productive activity with the actual level of (unwaged and waged) productive activity achieved during the 4 weeks postdischarge. These losses were converted to a monetary value by using market prices for labor, approximated by average pretax earnings (*13*).

The question we address is whether postdischarge SSIs independently affect costs. The specific cost outcomes we seek to explain are listed in online Appendix 1 (available from http://www.cdc.gov/ncidod/EID/vol12no05/05-1321_app1.htm). Adjustment was made for other factors believed to influence these cost outcomes (i.e., confounding factors); these included the type of surgical procedure, duration of surgery, American Society of Anesthesiologists score, wound class, number of coexisting conditions, length of hospital stay, whether patient was funded by the public sector or private insurance, admitting hospital, sex, age, ethnicity, patient's socioeconomic status (*14*), whether the patient was in waged employment, salary level and health-related quality of life as measured by the SF-12v2 Health Survey (available from http://www.sf-36.org/tools/sf12.shtml) scores at baseline and 4 weeks postdischarge. The complete set of explanatory variables available for analyses and the summary statistics are presented in Table 1 and online Appendix 2 (available at http://www.cdc.gov/ncidod/EID/vol12no05/05-1321_app2.htm). Because the outcome variables were continuous and linear, ordinary least squares regression was chosen to model the independent effect of SSI on cost outcomes (Table 2). See online Appendix 3 (available at http://www.cdc.gov/ncidod/EID/vol12no05/05-1321_app3.htm) for a description of the statistical analyses.

The mean age of the 449 patients included in the analyses was 63.65 years (SD 14.34), and 50.56% were women. The mean length of hospital stay for the sample was 7.8 days (SD 8.68, median 6 days, interquartile range 4–8). Thirty-eight of the 449 patients included in the study had a diagnosis of SSI postdischarge, which indicates an incidence of 8.46% for the 8-month period during which patients were recruited. A higher proportion of persons

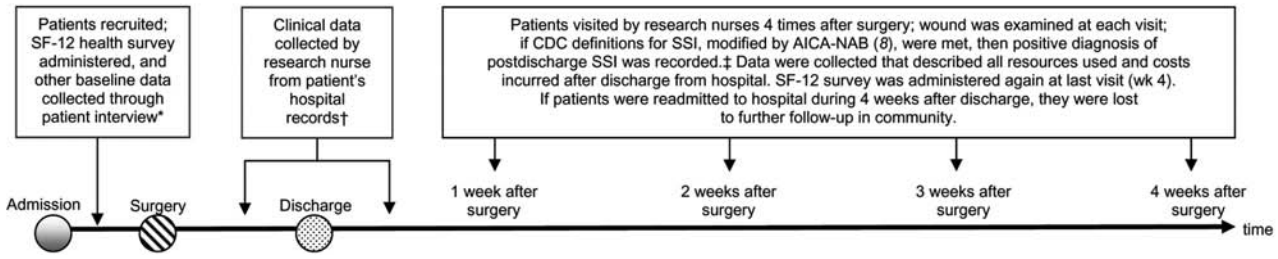

Figure. The timing and nature of data collection. *Interview questions available from author on request. †Types of data collected from patient hospital records available from author on request. ‡Variables collected from patient at each nurse visit are available from author on request. CDC, Centers for Disease Control and Prevention; SSI, surgical site infection; AICA-NAB, Australian Infection Control Association–National Advisory Board.

with SSI (18.24%) compared to those without SSI (2.43%) were readmitted to the hospital, but the mean lengths of stay of the readmitted persons were similar, 16.57 days versus 15.72 days, respectively. Summary statistics for all variables are included in Table 1 and in online Appendix 2, and the ICD-10 procedures for the 38 cases of SSI are described in online Appendix 4 (available at http://www.cdc.gov/ncidod/EID/vol12no05/05-1321_app4.htm).

No evidence was found of multicollinearity or interactions between variables. However, none of the outcome variables were normally distributed, and variance of the error term was not constant (i.e., heteroscedastic), so all models were estimated by using the Huber–White covariance matrix (*15*). Results of the ordinary least squares regressions are summarized in online Appendix 1. Strong statistical evidence shows that postdischarge SSI independently causes the following: 1.36 extra contacts with community-based services with increased costs of $47.78; 6.46 days of additional antimicrobial drug therapy with increased costs of $14.44; and an increase in total health service costs of AU $74 (US $57) when the costs of readmission to the hospital are excluded and AU $123 (US $94) when the costs of readmission to the hospital are included. The strength of the relationship between SSI and all other cost outcomes was not significant with the 95% confidence interval crossing zero for all other models.

Table 1. Demographic characteristics of included patients by surgical site infection (SSI)

	% (no.)	
Characteristic	No SSI (n = 411)	SSI (n = 38)
Age, y, mean (SD)	63.58 (14.41)	64.37 (13.72)
Socioeconomic score (1–100),* mean (SD)	35.67 (19.17)	40.37 (20.53)
Male	48.66 (199)	57.89 (22)
Recruiting hospital		
280-bed district hospital	35.04 (144)	47.37 (18)
712-bed teaching hospital	47.45 (195)	36.84 (14)
156-bed district hospital	16.79 (69)	15.79 (6)
Income		
Currently in waged employment	20.68 (85)	31.58 (12)
≤$50,000/y	12.41 (51)	23.68 (9)
>$50,000/y	2.68 (11)	5.26 (2)
Refused to answer	5.35 (22)	2.63 (1)
Education		
Left school at ≤15 y	60.83 (250)	63.16 (24)
Left school at 16–18 y	7.54 (31)	13.16 (5)
Some form of higher education	30.41 (125)	23.68 (9)
Ethnicity		
Caucasian	96.11 (395)	97.37 (37)
Aboriginal	0.24 (1)	0.00 (0)
Asian	0.24 (1)	0.00 (0)
Other	2.43 (10)	2.63 (1)
How patient was funded		
Public	91.97 (378)	94.74 (36)
Intermediate	6.81 (28)	5.26 (2)
Private	0.24 (1)	0.00 (0)

*See Jones and McMillan (*14*) for the scoring algorithm used.

Table 2. Cost outcomes*

Outcome	Mean (SD)	
	No SSI, n = 411	SSI, n = 38
Healthcare services		
No. contacts with hospital-based services in 4 wk PD	1.10 (1.68)	1.11 (1.43)
Cost of contacts with hospital-based services in 4 wk PD ($)	40 (60)	40 (52)
No. contacts with community-based services in 4 wk PD	1.85 (2.72)	3.13 (3.04)
Cost of contacts with community-based services in 4 wk PD ($)	62 (103)	105 (111)
No. tests/swabs	0.48 (1.43)	0.71 (1.27)
Costs of tests/swabs ($)	11 (35)	16 (28)
No. of days on antimicrobial drugs during 4 wk PD	0.96 (3.10)	6.76 (8.23)
Costs of antimicrobial drugs	2.16 (9.08)	14.47 (19.96)
Sum of all costs incurred by health care services, excluding costs of readmission ($)	115 (128)	176 (144)
Sum of all costs incurred by health care services, including costs of readmission ($)	417 (3050)	2,361 (8,811)
Production losses		
Patient production losses during 4 wk PD (min)	5,237 (5,488)	7,295 (6,349)
Monetary valuation of patient production losses ($)	1,895 (1,986)	2,640 (2,298)
Informal care givers production losses during 4 wk PD	1,630 (2,329)	2,863 (3,168)
Monetary valuation of Informal care giver production losses ($)	590 (843)	1,036 (1,146)
Private costs		
Time patient spent accessing hospital services (min)	169 (444)	184 (338)
Time patient spent accessing community-based services (min)	129 (410)	282.76 (528.14)
Total out-of-pocket expenditures during 4 wk PD ($)	5 (19)	4 (21)
SF-12 Physical Component Summary (enrollment)	39.15 (11.76)	37.63 (12.24)
SF-12 Mental Component Summary (enrollment)	50.37 (10.06)	48.87 (10.60)
SF-12 Physical Component Summary (wk 4)	39.03 (8.84)	37.68 (8.04)
SF-12 Mental Component Summary (wk 4)	53.92 (8.35)	52.06 (11.10)

*SSI, surgical site infection; PD, postdischarge; min, minutes of time.

Conclusions

These results support the view that most SSIs first appear after discharge from hospital, but we did not find any evidence that postdischarge SSI causes substantial economic costs even when costs are viewed from a societal perspective. These findings contradict Perencevich et al. (*5*), who found the economic cost of a case of SSI diagnosed after discharge was almost 50-fold the estimate we report here. Thus, what might explain this extreme discrepancy in attributed costs? The study designs and research methods differed. Compared to Perencevich et al. (*5*), we used more control variables (described in Tables 1 and Online Appendix 2 and listed below the table in Appendix 1). Might this extended set of control variables reduce bias from omitted variables and so reduce the cost attributed to SSI? Another factor might be the surveillance method. Perencevich et al. (*5*) used automated record screening that relied on accurate documentation of diagnostic, testing, or treatment codes and pharmacy records. This process resulted in 89 diagnoses among 4,571 patients, an incidence rate of 1.9%. For our study, patients were recruited before surgery and infection-control research-nurses visited the patients in their homes on 4 occasions after discharge, during which time the wound was examined and the definition of the Centers for Disease Control and Prevention definition, modified by the Australian Infection Control Association Inc., was applied (*8*). This method yielded a much higher infection rate of 8.38%. One interpretation is that the surveillance method used by Perencevich et al. was not sensitive to all cases of postdischarge SSI. Instead, only those that generated certain data items in the downstream electronic records were flagged, and these may have been the most serious cases of SSI that generated the greatest costs. This theory might be supported by the higher rate of readmission among the patients with cases of SSI in the Perencevich data (34%) compared to the rate in our study (18%).

Of course, other factors may have an influence, such as the case mix and socioeconomic characteristics of the participants, the costs of the inputs to healthcare services (i.e., salaries for doctors and nurses), consumer preferences (i.e., for more or less postdischarge care), and predefined care protocols.

Also, our data only describe a 4-week period after surgery and not the 8-week period considered by Perencevich et al. (*5*). We recommend that readers interpret our results carefully but nevertheless suggest that the economic costs of SSIs that occur after hospital discharge are real but not substantial.

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Historical Lassa Fever Reports and 30-year Clinical Update

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Five cases of Lassa fever have been imported from West Africa to the United States since 1969. We report symptoms of the patient with the second imported case and the symptoms and long-term follow-up on the patient with the third case. Vertigo in this patient has persisted for 30 years.

Lassa fever is a viral hemorrhagic fever caused by a rodentborne arenavirus that is endemic in West Africa. In 2004, the Centers for Disease Control and Prevention (CDC) reported a fatal case of Lassa fever in New Jersey (*1*). CDC noted that ≈20 imported cases of Lassa fever had been seen outside West Africa. Five patients with imported disease have been hospitalized in the United States (Table). We report the signs and symptoms of the second patient in this group of 5 patients and the signs and symptoms and long-term follow-up of the third patient, both aid workers who became ill in 1975 while serving in Sierra Leone. Their clinical courses were complicated by severe neurologic dysfunction, including unilateral sensorineural deafness and vertigo.

Case 1

In February 1975, a 26-year-old American aid worker in Sierra Leone was hospitalized with severe abdominal pain. No cause was determined, and she was discharged. In March 1975, watery diarrhea, fever, chills, headache, myalgias, arthralgias, and conjunctival injection developed. She was hospitalized, and physical examination showed posterior cervical, axillary, and inguinal lymphadenopathy. She was empirically treated for malaria and amebiasis.

Nevertheless, fever persisted, she lost 2.7 kg, and pleuritic chest pain developed. In April 1975, she was air evacuated and admitted to a hospital in Washington, DC. Although she was afebrile, generalized lymphadenopathy was still present, and a chest radiograph showed left-sided pleural effusion. Thoracentesis fluid was remarkable for eosinophilia, and examinations of blood showed 3%–35% peripheral eosinophilia. Knott's preparation of blood showed 3 sheathed microfilariae with nuclei extending into the tail, presumed to be *Loa loa*. A cervical lymph node biopsy showed follicular hyperplasia.

She was convalescing in the hospital when suddenly, while speaking on the telephone, she lost hearing unilaterally. An audiogram demonstrated unilateral sensorineural deafness. A serum specimen collected in May 1975 was sent to CDC, where an indirect fluorescent antibody (IFA) titer of 256 was demonstrated against Lassa fever virus (P. Rollin, pers. comm.). She was discharged with residual unilateral deafness.

Case 2

In December 1975, abdominal cramps, nausea, vomiting, diarrhea, fatigue, headaches, retroorbital pain, aching shoulders, and severe low back pain developed in a 43-year-old American aid worker in Sierra Leone.[1] Her aching progressed to total body pain, which she described as "severe pain in her bones, as if they were breaking" (from patient's medical chart). Her symptoms persisted, and in February 1976, nocturnal fevers and sweats developed. She experienced dizziness and syncope and was hospitalized. She was hypotensive with blood pressure as low as 70/40 mm Hg (compared to 120/80 mm Hg in June 1975) and had insomnia. She was empirically treated for malaria and discharged. Her symptoms reappeared, accompanied by persistent vomiting, shooting pain in the right ear, neck pain, paresthesias, and alopecia. She lost 4 kg. In March 1976, she was air evacuated and admitted to a hospital in Washington, DC.

During her hospitalization in Washington, she was afebrile. However, fatigue, headache, neck pain, nausea, low back pain, and insomnia persisted. She had costochondral and diffuse abdominal tenderness and ecchymoses at intramuscular injection sites (antiemetics). She was unable to read for more than a few minutes, as her eyes would tire and begin to hurt. She experienced dysmorphopsias, difficulty with hearing, severe depression, and numerous episodes of lightheadedness, unsteadiness, dizziness, and vertigo. Vertigo occurred in both supine and standing positions up to 5 times per day. Although she was hypotensive, she was not orthostatic. Neurologic examination found left-sided facial weakness, right-sided Babinski reflex, and the Weber test lateralized to the left. Audiometry and positional and caloric nystagmography results were unremarkable.

A serum specimen obtained on March 1 showed an IFA titer of 64 against Lassa virus. Lassa virus was recovered

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[1]Portions of this patient's clinical signs and symptoms were originally published in Zweighaft et al. (*2*).

Table. Patients with imported Lassa fever who were hospitalized in the United States*

Patient no.	Year of import	From	To	Clinical manifestations
1	1969	Nigeria	New York, NY	Fever, malaise, headache, nausea, sore throat, epigastric/right upper quadrant tenderness, pleural effusion, facial/cervical edema, dysphagia, elevated transaminases, cough, dyspnea, pulmonary infiltrates, epiglottal edema, lethargy, nystagmus, lightheadedness, dizziness without vertigo, ataxia, alopecia (*2*)
2	1975	Sierra Leone	Washington, DC	Abdominal pain, diarrhea, fever, headache, myalgia, arthralgia, conjunctival injection, lymphadenopathy, weight loss, pleuritic chest pain, pleural effusion, unilateral deafness
3	1976	Sierra Leone	Washington, DC	Abdominal cramps, nausea, vomiting, diarrhea, fatigue, headache, retroorbital pain, neck/back pain, paresthesias, right ear pain, fever, vertigo, syncope, dysmorphopsias, alopecia, weight loss, ecchymoses, insomnia, depression, hypotension, left-sided facial weakness, right-sided Babinski reflex, Weber test lateralized to the left (*3*)
4	1989	Nigeria	Chicago, IL	Shaking chills, fever, sore throat, myalgia, headache, dysphagia, bloody diarrhea, elevated transaminases, hypotension, adult respiratory distress syndrome, death (*4*)
5	2004	Sierra Leone and Liberia	Trenton, NJ	Chills, fever, sore throat, diarrhea, back pain, adult respiratory distress syndrome, death (*1*)

*Patients 1–4 are US citizens; patient 5 is a Liberian national.

from a March 3 urine specimen. On March 10, a serum specimen demonstrated a complement fixation antibody titer of 16, a 4-fold rise compared to a titer <4 in a February 25 specimen drawn in Sierra Leone.

Although her vertigo persisted, she became normotensive (120/80 mm Hg) on March 28, 1976, and was discharged. However, during the next 30 years, she continued to experience fatigue, generalized weakness, headache, insomnia, depression, dysmorphopsias, paresthesias, lightheadedness, dizziness and syncope, and labile hypotension. She describes "fatigue so severe that I have no energy for days," "staggering when getting up," "inability to produce words at times," and "spells of loss of consciousness" (up to 15 minutes in duration, as noted by her husband). In 1992, a magnetic resonance imaging scan of the brain demonstrated periventricular hyperintense signals. As of February 2006, her symptoms persist.

Conclusions

Auditory or vestibular dysfunction may develop in patients with Lassa fever, and tinnitus, autophony, hearing loss, dizziness, vertigo, nystagmus, and ataxia have been reported (*3,4*). In their review of a 1989 nosocomial Lassa fever outbreak in a Nigerian hospital, Fisher-Hoch et al. (*3*) noted a high fever in the index patient, who was taken to surgery on February 25. The patient bled profusely and died later that night. The surgical nurse and a student nurse who washed blood-soaked cloths both became ill with febrile illnesses on March 7. Both became serologically positive for Lassa fever virus. The surgical nurse was traced to her village, where she was found to be almost totally deaf and severely ataxic.

Onset of deafness among patients with Lassa fever is a feature of the convalescent phase rather than the acute phase of the illness (*4*). Deafness was first reported as a complication of Lassa fever by White (*5*) and Henderson (*6*) in 1972. White noted that during a 1970 nosocomial hospital outbreak in Jos, Nigeria, deafness occurred in 4 of 23 hospitalized patients; a fifth patient reported intermittent tinnitus, and 3 patients experienced dizziness.

Among the now 24 reported patients with imported Lassa fever worldwide (1969–2004, Appendix Table), our 26-year-old aid worker is the only patient whose clinical course has been complicated by sensorineural deafness. Our second patient's clinical course has been remarkable for an array of acute and chronic neurologic and neuropsychiatric complications, including left-sided facial weakness, right-sided Babinski reflex, headache, paresthesias, vertigo, syncope, dysmorphopsias, fatigue, insomnia, and depression. Rose (*7,8*) reported a 1955–1956 outbreak of encephalomyelitis in Sierra Leone, which may represent the earliest recorded clinical description of Lassa fever; remarkably, vertigo developed in 30 of his 45 patients. Solbrig and McCormick (*9*) reported that neuropsychiatric sequelae of Lassa fever have included sleep disorders (e.g., insomnia), asthenia, multiple somatic complaints, psychosis, hallucinations, personality disorders, severe adjustment reactions, dementia, mania, and depression. Finally, our patient's ongoing labile hypotension may represent Lassa fever–induced damage to the brain stem with resultant autonomic dysfunction. Since our patient's array of persistent neurologic and neuropsychiatric symptoms have not changed, improved, or progressed since her episode of Lassa fever, we believe that they all may represent sequelae of Lassa fever–induced damage to the brain.

Dr Macher is a 30-year veteran of the US Public Health Service. He retired in the summer of 2005 and currently advocates for indigent inmates' access to the standard of care. His research interests include the effects of privatization on correctional health care and postrelease access to continuity of care.

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Appendix Table. Patients with imported Lassa fever, worldwide, 1969–2004*

Year of import	From	To	Occupation	Clinical outcome
1969	Nigeria	United States	Nurse	Survived
1971	Sierra Leone	United Kingdom	Nurse	Survived
1971	Sierra Leone	United Kingdom	Physician	Survived
1972	Sierra Leone	United Kingdom	Nurse	Survived
1974	Nigeria	Germany	Physician	Survived
1975	Nigeria	United Kingdom	Physician	Died
1975	Sierra Leone	United States	Aid worker	Survived
1976	Sierra Leone	United States	Aid worker	Survived
1976	Nigeria	United Kingdom	Engineer	Survived
1980	Upper Volta	Netherlands	Aid worker	Survived
1981	Nigeria	United Kingdom	Teacher	Survived
1982	Nigeria	United Kingdom	Diplomat	Survived
1984	Sierra Leone	United Kingdom	Geologist	Survived
1985	Sierra Leone	United Kingdom	Nurse	Survived
1987	Sierra Leone/Liberia	Israel	Engineer	Survived
1987	Sierra Leone	Japan	Engineer	Survived
1989	Nigeria	Canada	Agricultural specialist	Survived
1989	Nigeria	United States	Engineer	Died
2000	Cotê d'Ivoire/Burkina Faso/Ghana	Germany	Student	Died
2000	Sierra Leone	United Kingdom	Peacekeeper	Died
2000	Nigeria	Germany	Unknown	Died
2000	Sierra Leone	Netherlands	Physician	Died
2003	Sierra Leone	United Kingdom	Peacekeeper	Survived
2004	Sierra Leone/Liberia	United States	Businessman	Died

*A fully referenced version of this appendix table is available online from http://www.cdc.gov/ncidod/EID/vol12no05/05-0052_app.htm

director of the private Parasitology Laboratory of Washington, Inc., and director of the Travelers Medical Service of Washington. His research interests include intestinal parasites and febrile diseases.

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Hantavirus in African Wood Mouse, Guinea

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Hantaviruses are rodentborne, emerging viruses that cause life-threatening human diseases in Eurasia and the Americas. We detected hantavirus genome sequences in an African wood mouse (*Hylomyscus simus*) captured in Sangassou, Guinea. Sequence and phylogenetic analyses of the genetic material demonstrate a novel hantavirus species, which we propose to name "Sangassou virus."

Hantaviruses, family *Bunyaviridae*, are emerging viruses that cause 2 life-threatening human zoonoses: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS, also known as hantavirus cardiopulmonary syndrome). The virus genome consists of 3 segments of negative-stranded RNA; the large (L) segment encodes viral RNA-dependent RNA polymerase, the medium (M) segment encodes glycoprotein precursor, and the small (S) segment encodes nucleocapsid protein. In contrast to other members of the *Bunyaviridae*, hantaviruses are not transmitted by arthropods but are spread by aerosolized excreta of rodents of the family *Muridae*, their natural hosts (*1–4*).

Hantaviruses have a strong association with certain reservoir host species. Phylogenetic analyses have divided hantaviruses into 3 major groups according to 3 subfamilies of their natural hosts. Hantaan virus (HTNV), Seoul virus (SEOV), and Dobrava virus (DOBV), which cause HFRS in Asia and Europe, are examples of *Murinae*-associated viruses. Puumala virus (PUUV), which causes a mild form of HFRS in Europe, and the less pathogenic Tula virus (TULV) are *Arvicolinae*- associated hantaviruses. In 1993, Sin Nombre virus (SNV) was discovered in the United States as the first member of the third group, *Sigmodontinae*-associated hantaviruses. SNV from North America and Andes virus (ANDV) from South America are the most prominent examples of viruses causing HPS (*3*,*5*).

Hantaviruses cause human diseases predominantly in Asia, Europe, and the Americas. Few studies have considered hantaviruses in Africa; such reports originated from serologic surveys of human populations. In this study, we report detection and initial genetic characterization of the first indigenous African hantavirus detected in an African wood mouse (*Hylomyscus simus*) in Sangassou, Guinea.

The Study

In a survey for rodentborne hemorrhagic fever viruses, 612 small rodents representing 17 different genera (most abundant were *Mastomys* [n = 325], *Praomys* [n = 95], and *Nannomys* [n = 83]) were trapped in Guinea from 2002 to 2004 and screened for hantavirus RNA by reverse transcription–polymerase chain reaction (RT-PCR). We used a molecular genetic approach to screen the rodent population because hantavirus RNA (as shown for SNV) can be amplified from the blood of persistently infected mice by RT-PCR over a long period (*6*). For this purpose, we developed a nested RT-PCR assay to detect currently known and possible novel members of the genus *Hantavirus*. The assay was based on degenerated primers (HAN-L-F1: 5′-ATGTAYGTBAGTGCWGATGC-3′ and HAN-L-R1: 5′-AACCADTCWGTYCCRTCATC-3′ for primary PCR, HAN-L- F2: 5′-TGCWGATGCHACIAARTGGTC-3′ and HAN-L-R2: 5′-GCRTCRTCWGARTGRTGDGCAA-3′ for nested PCR) designed from an alignment of all available nucleotide sequences of the highly conserved L segment. For the RT-PCR, total RNA was extracted from wild-trapped rodent blood (preserved in liquid nitrogen) with the Blood RNA kit (Peqlab, Erlangen, Germany) and reverse transcribed with random hexamers as primers.

A sample (designated SA14) obtained from 1 of 4 investigated African wood mice (*H. simus*) generated an L segment–derived PCR product of expected size. This rodent was trapped in January 2004 in a forest habitat near the village of Sangassou, near Macenta, Guinea (8°36′49″N, 9°28′27″W). Its karyotype was determined (2n = 48, fundamental no. = 74, autosomal fundamental no. = 70) and the complete cytochrome *b* gene was sequenced and compared with the genes of other *Hylomyscus* species recognized in the most recent revision of the genus (*7*) (GenBank accession nos. DQ212188 and DQ078229-DQ078245).

The 412-nucleotide (nt) sequence of the first PCR product was determined by amplification, cloning, and sequencing of overlapping fragments generated by 2

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seminested PCRs (GenBank accession no. DQ268652). Additional S and M segment–specific nested PCR assays were developed to further characterize the novel virus. PCR fragments of 837 nt and 694 nt could be analyzed (GenBank accession nos. DQ268650 and DQ268651, respectively).

The Table shows nucleotide sequence identity comparisons between SA14 and other members of the genus *Hantavirus*. *Murinae*-associated hantaviruses (HTNV, DOBV, SEOV) showed the highest similarity to the SA14 sequence in all 3 genomic segments (71.3%–77.1% for S, 72.9%–77.9% for M, and 72.3%–75.9% for L). This similarity is consistent with the evolutionary relationship of their putative hosts. On the amino acid level, corresponding sequences of deduced viral proteins showed highest similarity with those of other *Murinae*-associated hantaviruses (81.7%–88.5% for S, 82.2%–89.6% for M, and 85.4%–87.5% for L). The amino acid sequence divergence between SA14 and most related hantaviruses corresponds to that typically found between different virus species, e.g., SNV and ANDV.

The S-, M-, and L-segment–derived nucleotide sequences of SA14 were subjected to maximum likelihood (ML) and neighbor-joining (NJ) phylogenetic analyses with available nucleotide sequences of other *Murinae*-associated hantaviruses. PUUV, TULV, SNV, and ANDV sequences were used as outgroups. In the S segment ML phylogenetic tree (Figure), SA14 clustered with other *Murinae*-associated viruses. As expected, 3 clades were formed by members of the 3 established hantavirus species (HTNV, DOBV, and SEOV). Within this well-supported cluster, the SA14 sequence is most closely related to the DOBV clade. The M segment analysis showed an identical placement of SA14 with strong statistical support (PUZZLE [*8*] and bootstrap values above the threshold value of 70%, data not shown). In L-segment phylogeny, the resolution of the tree was decreased. The SA14 L sequence did not join with statistical support any of the 3 groups but formed a fourth clade within the cluster of *Murinae*-associated hantaviruses. (PUZZLE and bootstrap values above the threshold for the placement of SA14 within the *Murinae*-associated viruses but <50% in both analyses for its clustering with any of these viruses, data not shown).

Conclusions

Extended fragments of novel hantavirus S, M, and L genome segments were recovered from an arboreal African rodent. They clearly represent genetic material of a novel hantavirus species because their amino acid sequence is significantly (≈15%) divergent from those of other hantaviruses, they form a distinct clade in phylogenetic trees, and they were detected in a rodent species previously not recognized as a natural host of hantaviruses. We propose to name the new species Sangassou virus (SANGV) after the locality where it was detected.

Although hantaviruses are emerging viruses circulating in Asia, Europe, and the Americas, our study represents the first genetic evidence for hantaviruses in Africa. Suspected human hantavirus infections have been reported in various African countries (*10*–*13*). Most of these are seroepidemiologic studies reporting antibodies reacting with HTNV antigen. However, *Apodemus agrarius*, the natural host of HTNV, is not found in Africa. Based on the putative cross-reactivity of antigens from HTNV, SANGV, and other *Murinae*-associated viruses, human infections, at least in tropical forest parts of Africa where *Hylomyscus* species are prevalent, could be caused by SANGV or other *Murine*-associated hantaviruses.

To our knowledge, 1 case of HFRS has been reported in central Africa (*14*). Although HFRS is not a known disease in West or central Africa, one cannot ignore the potential pathogenicity of SANGV or other African hantaviruses. HFRS may be confused with other severe diseases (leptospirosis, rickettsiosis, other viral hemorrhagic fevers, plague, severe pneumonia, sepsis) or may be unrecognized because of poor health care. One should remember that

Table. Similarity of (% identity with SA14 partial sequences) SA14 partial S, M, and L segment sequences with those of other hantaviruses*†

	S segment		M segment		L segment	
Hantavirus	nt	aa	nt	aa	nt	aa
$HTNV_{76\text{-}118}$	71.3	81.7	77.9	89.6	72.3	86.1
$SEOV_{80\text{-}39}$	75.8	82.4	72.9	82.2	75.9	87.5
$DOBV_{SK/Aa}$	77.1	88.5	77.6	89.6	73.0	85.4
$PUUV_{CG1820}$	61.4	61.1	62.1	61.4	69.6	72.2
$TULV_{Moravia}$	62.0	62.3	62.5	62.7	65.2	72.2
$SNV_{NM\ H10}$	63.2	62.3	60.8	62.3	68.9	72.2
$ANDV_{Chile\text{-}R123}$	62.0	62.7	65.5	62.7	68.6	72.9

*S, small; M, medium; L, large; HTNV, Hantaan virus; SEOV, Seoul virus; DOBV, Dobrava virus; PUUV, Puumala virus; TULV, Tula virus; SNV, Sin Nombre virus; ANDV, Andes virus.
†837 nucleotides (nt) of the S segment (positions 394–1230), 694 nt of the M segment (positions 2281–2974), and 412 nt of the L segment (positions 2956–3367) and the deduced amino acid (aa) sequences (279 aa, position 120–398 of the nucleocapsid protein; 231 aa, positions 748–978 of the glycoprotein precursor; 137 aa, positions 974–1110 of the viral RNA-dependent RNA polymerase) have been compared. Fragment positions were defined according to complete sequences of HTNV strain 76-118 (GenBank accession nos. NC_005218, NC_005219, and NC_005222).

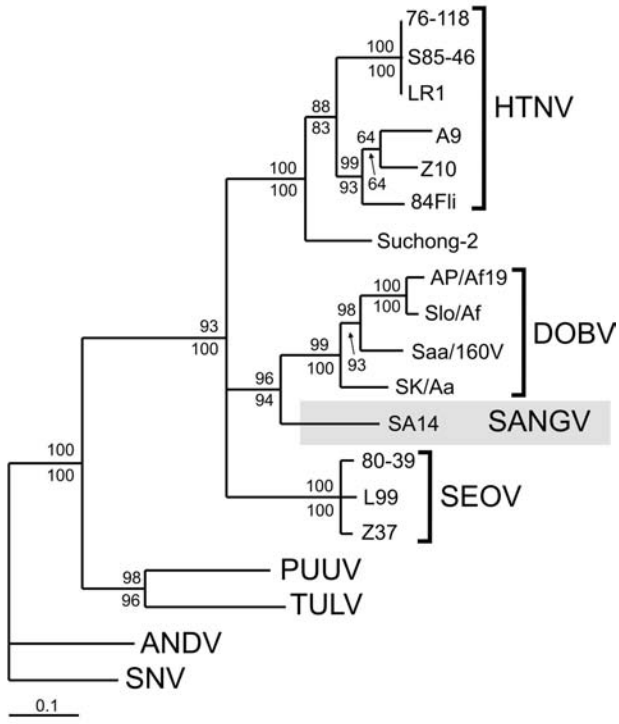

Figure. Maximum likelihood phylogenetic tree of hantaviruses showing the placement of SA14 (Sangassou virus [SANGV], indicated by gray shading). Partial S segment genome sequences (837 nucleotides, positions 394–1230) were used to calculate the tree with TREE-PUZZLE program (*8*). The Tamura-Nei evolutionary model was used; the values above the branches represent PUZZLE support values. The values below the branches represent bootstrap values of the corresponding neighbor-joining tree computed with PAUP* program (*9*) using 10,000 bootstrap replicates. The scale bar indicates an evolutionary distance of 0.1 nucleotide substitutions per position in the sequence. HTNV, Hantaan virus; DOBV, Dobrava virus; SEOV, Seoul virus; PUUV, Puumala virus; TULV, Tula virus; ANDV, Andes virus; SNV, Sin Nombre virus.

HPS and *Sigmodontinae*-associated hantaviruses were not recognized until 1993, even in such a highly developed country as the United States.

Further studies are needed to verify the presence and distribution of hantaviruses in Africa and their potential impact on human health. These studies should focus on areas with forest activities, such as logging, which may bring humans into contact with viral reservoirs (*15*). Our data justify inclusion of hantavirus infection in the differential diagnosis of patients from Africa with unexplained febrile nephropathies or noncardiogenic pulmonary edema.

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Rickettsia felis in Fleas, Western Australia

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This study is the first confirmation of *Rickettsia felis* in Australia. The organism was identified from 4 species of fleas obtained from dogs and cats in Western Australia, by using polymerase chain reaction amplification and DNA sequencing of the citrate synthase and outer membrane protein A genes.

Several rickettsial diseases have been documented in Australia, namely, Queensland tick typhus (*Rickettsia australis*), scrub typhus (*Orientia tsutsugamushi*), murine typhus (*R. typhi*), and more recently, Flinders Island spotted fever (*R. honei*), as well as the closely related Q fever (*Coxiella burnetii*) and cat scratch fever (*Bartonella henselae*) (*1,2*). Cases of murine typhus have been reported in Western Australia (WA) since 1927, and a serologic survey provided evidence that members of the closely related spotted fever group (SFG) rickettsiae are also present in the state (*3,4*).

R. felis is a newly discovered species within the SFG; it is transmitted by fleas, which makes it unique within the biogroup. The species was first detected in the cat flea, *Ctenocephalides felis*, and subsequently has been determined to cause human disease in a number of countries (*5–8*). A recent study in New Zealand provided the first report of the organism in Oceania (*9*). Infected domestic and wild animals may not exhibit clinical disease and act as reservoirs of infection for humans. No definitive reports of the organism have been made in Australia, however, a study of cat fleas that used polymerase chain reaction and restriction fragment length polymorphisms of the amplification products (PCR-RFLP), provided strong evidence that *R. felis* exists (*10*). Our study aimed to confirm the presence of *R. felis* in Australia and to determine the distribution of the organism in WA. This study was approved by the Murdoch University Animal Ethics Committee.

The Study

Samples were collected from 8 regional centers throughout WA: Esperance, Albany, Augusta, Manjimup, Busselton, Bunbury, Pinjarra, and Geraldton. Veterinarians from each site collected fleas from dogs and cats, preserved them in 70% ethanol, and sent them to Murdoch University for identification and analysis. The fleas were identified by using light microscopy. For each of the sampled 116 dogs and 43 cats, 1–5 fleas were pooled to increase the likelihood of finding rickettsial DNA. DNA was extracted from each flea pool by using a Qiagen QIAmp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Initial PCR targeted the citrate synthase (*gltA*) gene, which is conserved across the genus *Rickettsia*. Primers and PCR conditions were sourced from the literature (*11*). Samples positive by the initial screening PCR were then selected for a second round of PCR targeting the outer membrane protein A gene (*ompA*), which is specific for spotted fever group (SFG) rickettsiae (*12*), and thus distinguishes *R. typhi* and *B. henselae*, which are also found in fleas and would be detected with the *gltA* PCR, from *R. felis*. PCR conditions were validated and optimized by using *R. typhi*- and *R. felis*-positive controls.

All PCR products were separated on a 1% agarose gel at 86 V for 30 min and visualized under UV light. Eight *C. felis* samples that were positive for both *gltA* and *ompA* were sequenced by using the *gltA* primers. Two of these samples were also sequenced by using *ompA* primers. The products of the PCR were extracted from the agarose for sequencing by using the Qiagen gel extraction kit (Qiagen) according to the manufacturer's recommendations. Purified PCR products were sequenced by using the Big Dye version 3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 373 automatic sequencer and were compared to those of previously characterized rickettsiae in GenBank by using BLAST (available from http://www.ncbi.nlm.nih.gov) analysis.

Conclusions

A total of 368 fleas collected from 43 cats and 116 dogs were pooled into 165 flea pools (mixed infections from 6 animals meant 6 more flea pools than the total number of animals). Four different species of flea were identified: *C. felis* (49 from 38 cats and 241 from 99 dogs), *C. canis* (12 from 7 dogs), *Echidnophaga gallinacea* (4 from 3 cats and 57 from 16 dogs), and *Spilopsyllus cuniculi* (5 from 2 cats). Six dogs had a mixed population of fleas; 4 of these had *C. felis* and *E. gallinacea*, and 2 had *C. felis* and *C. canis*.

Forty-two (36%) of the 116 flea pools from dogs were positive for both the *gltA* and *ompA* genes. Similarly, 14 (33%) of 43 flea pools from cats were positive for both genes. Notably, positive samples were obtained from all the locations in the study, indicating widespread distribution throughout the state (Table).

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Table. Flea species positive for rickettsiae from dogs and cats in Western Australia*

Location	Animal	No. animals	No. flea pools	Flea species	No. *gltA*+*ompA*+ (%)	Genes sequenced†
Broome	Dog	4	4	*Echidnophaga gallinacea*	2 (50)	ns
Geraldton	Cat	17	14	*Ctenocephalides felis*	6 (43)	ns
			3	*E. gallinacea*	1 (33)	ns
	Dog‡	38	30	*C. felis*	14 (46)	ns
			10	*E. gallinacea*	2 (20)	ns
Pinjarra	Cat	1	1	*C. felis*	0 (0)	
	Dog	7	7	*C. felis*	3 (43)	*gltA*
Manjimup	Cat	4	4	*C. felis*	1 (25)	ns
	Dog	6	6	*C. felis*	3 (50)	*gltA*
Bunbury	Cat	5	5	*C. felis*	2 (40)	ns
	Dog	6	6	*C. felis*	3 (50)	*gltA*
Busselton	Cat	10	8	*C. felis*	3 (38)	ns
			2	*Spilopsyllus cuniculi*	0	
	Dog§	33	28	*C. felis*	6 (21)	*gltA*, *ompA*
			7	*C. canis*	0	
			1	*E. gallinacea*	0	
Augusta	Cat	1	1	*C. felis*	0	
	Dog	2	2	*C. felis*	1 (50)	*gltA*
Albany	Dog	10	10	*C. felis*	5 (50)	*gltA*
Esperance	Cat	5	5	*C. felis*	3 (60)	ns
	Dog¶	10	10	*C. felis*	3 (30)	ns
			1	*E. gallinacea*	0	

*Fleas were collected from 116 dogs and 43 cats. 6 animals were co-infected with 2 different species of fleas as indicated.
†ns, no sequencing performed on this sample; *gltA*–*gltA* segment sequenced; *ompA*–*ompA* gene segment sequenced.
‡Two dogs had *C. felis* and *E. gallinacea.*
§One dog had *C. felis* and *E. gallinacea*, 2 dogs had *C. felis* and *C. canis.*
¶One dog had *C. felis* and *E. gallinacea*; *gltA*, citrate synthase gene; *ompA*, outer membrane protein A gene.

Of the 8 samples from *C. felis* positive for both *gltA* and *ompA* genes that were sequenced by using the *gltA* primers, all sequences matched the *gltA* gene from *R. felis* (99% similarity). Of the 2 samples that were also sequenced by using *ompA* primers, the sequences matched the *R. felis ompA* gene (100% similarity).

Our study demonstrates that *R. felis* is present in multiple sites in WA and was conclusively present in 1 of the 4 flea species collected (*C. felis*). The results obtained from 2 rounds of PCR are highly indicative of *R. felis* infection in *E. gallinacea* also. Because *C. felis* has the highest rate of infection and is prevalent, highly mobile, and nonspecific in its choice of hosts (including humans), it is likely to be the most important vector of the organism. *C. canis* has been identified as a vector of *R. felis* (*13*); however, this finding was not supported by our study. The presence of *R. felis* in *E. gallinacea* has been previously reported (*14*). To our knowledge, this is the first time a rickettsia has been detected from *S. cuniculi,* which could also be a potential vector for *R. felis.*

The significance of *R. felis* as a cause of human disease in WA has not yet been determined. Because of the often transient and nonspecific symptoms of rickettsioses, infections may not be readily detected. A serologic survey conducted in the Kimberley region of WA (*10*) showed evidence of scrub typhus and an SFG rickettsia, but no further work has identified the specific organism responsible for the latter**.** Another serologic study of 866 people throughout southwest WA showed evidence of infection with *R. typhi* (0%–1%) and another undetermined SFG rickettsia (3%–13%). During the same study, fleas were collected from cats and dogs in Perth and screened for rickettsiae by using PCR-RFLP of the *gltA* gene; the results provided evidence for the existence of *R. felis*. However, no sequencing data confirmed its presence (*4*).

The results from the current study showed that the *gltA* gene from all the sequenced samples most closely matched the *gltA* gene in the species *R. felis*. The identity of the sequenced samples as *R. felis* was confirmed by the *ompA* gene sequences. Therefore, the other samples shown to be positive for SFG rickettsiae in the PCR screening process are probably also *R. felis.*

This study has confirmed the presence of *R. felis* in WA; consequently, this rickettsial disease should be included as a differential diagnosis for influenzalike illnesses in persons who own or work with companion animals.

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Mr Schloderer completed a Bachelor of Science at Murdoch University. This work formed part of his research which concentrated on investigating *Rickettsia* spp. in companion animals in Western Australia.

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Heterogeneity among *Mycobacterium ulcerans* Isolates from Africa

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Mycobacterium ulcerans causes Buruli ulcer, an ulcerative skin disease in tropical and subtropical areas. Despite restricted genetic diversity, mycobacterial interspersed repetitive unit–variable-number tandem repeat analysis on *M. ulcerans* revealed 3 genotypes from different African countries. It is the first time this typing method succeeded directly on patient samples.

Buruli ulcer (BU), the third most common mycobacterial disease after tuberculosis and leprosy, is a major health problem in several West and Central African countries (*1*). Although endemic in Central America and subtropical climates of Southeast Asia and Australia, countries in Africa in the past decade have recorded increased incidence rates in some communities exceeding that of tuberculosis (*2*).

Mode(s) of transmission, natural reservoir(s) and other key aspects of the epidemiology of BU are not fully understood, a situation partly complicated by an apparent lack of genetic diversity of *Mycobacterium ulcerans,* as shown by several independent genetic markers (*3*–*6*). Conventional and molecular data suggest that *M. ulcerans* is an environmental pathogen because of the selective association of BU-endemic foci with wetlands and overflowed river banks and the detection of *M. ulcerans*–specific sequences in water, mud, aquatic insects, and plants (*7*–*9*). Specific reservoirs of the etiologic agent cannot be definitively assigned; however, we have cultivated *M. ulcerans* from a single aquatic insect from Benin (*10*).

Extensive molecular typing of *M. ulcerans* isolates recovered from patients in many endemic foci has been undertaken to further the understanding of the epidemiology of BU. A set of robust genotyping methods has already been applied to *M. ulcerans*: IS*2404* restriction fragment length polymorphism (*11*), amplified fragment length polymorphism analysis (AFLP) (*12*), multilocus sequence typing (*13*), variable-number tandem repeat (VNTR) (*3*), mycobacterial interspersed repetitive unit (MIRU)–VNTR (*6*), IS*2426* polymerase chain reaction (PCR) (*5*), and IS*2404*-Mtb2 PCR (*4*). All methods, except AFLP, resulted in geographically related genotypes for China, Japan, Mexico, Suriname, French Guiana, Malaysia, Papua New Guinea II and Papua New Guinea III, Australia Victoria, Australia Queensland, and Africa. Current typing methods have established a striking geographic and temporal homogeneity in African isolates from Angola, Benin, Democratic Republic of Congo (DRC), Ghana, Côte d'Ivoire, and Togo (*3*–*6*). Even *M. ulcerans* cultured from the insect collected in Benin showed an identical African genotype (*6*). Recently, however, Hilty et al., using a VNTR typing method and sequence analysis, described 3 genotypes in Ghana (*14*). The development of more discriminating typing methods may unravel the source and mode of transmission of *M. ulcerans* and other epidemiologic aspects of BU.

Improved understanding of the molecular biology of *M. ulcerans* will likely help elucidate observed differences in clinical manifestations. Reported disease recurrence rates vary from 6% to >20% (*15*). To what degree this recurrence is attributable to exogenous reinfection or dissemination of the pathogen from previous lesions is unknown. The relative contribution of variations in pathogen and host factors to progression and severity of disease likewise remains obscure.

We report the first evidence of genetic diversity in *M. ulcerans* samples from 3 African countries: DRC, Sudan, and Uganda. Previously, we identified tandem repeat loci, MIRUs (*6*), and VNTRs (*3*) in the genome of *M. ulcerans*. A selection of these MIRUs and VNTRs were used in this study to analyze *M. ulcerans* extracts from tissue specimens from Benin, Togo, Gabon, Uganda, and Sudan, and from previous isolates from patients from Cameroon, DRC, Uganda, and Congo-Brazzaville (Table 1). Results were compared with those of a geographically diverse collection (n = 39) that were typed in our previous study (*6*).

The Study

To investigate the MIRU polymorphism, whole genomic DNA was prepared from bacterial cultures or clinical specimens. The specimens were tissue fragments from patients with nonulcerated (plaques and edematous forms) or ulcerated forms. DNA extraction from pure cultures was performed by heating the colonies in Tris-EDTA at 95°C for 10 minutes. Clinical specimens from laboratory-

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Table 1. MIRU-VNTR profiles of *Mycobacterium ulcerans* and origin of specimens (BK no.) or culture isolates*

ITM no./loci†	1‡	6‡	9‡	33‡	Genotype	Origin	Ziehl-Neelsen staining§	Year¶
5142	1	1	1	2	Victoria	Victoria, Australia		1967
9540	1	1	1	3	Southeast Asia	Queensland, Australia; PNG; Malaysia		1978
98-0912, 8756	1	2	1	3	Asia	China, Japan		1998
BK03-0621	2	1	1	3	PNGII	PNG	3+	2003
BK02-2487	2	1	1	1	PNGIII	PNG	1+	2002
BK04-0296	2	1	1	1		PNG	1+	2004
842	NA	1	2	1	Suriname	Suriname		1984
7922	2	2	2	1	French Guiana	French Guiana		1990
5114	1	2	2	1	Mexico	Mexico		1953
5116	1	2	2	2	Central African Congo River Basin	Maniema, DRC		1962
9099	1	2	2	2		Maniema, DRC		1964
5150	3	1	1	3	Atlantic Africa	Bas-Congo, DRC		1962
94-0662	3	1	1	3		Côte d'Ivoire		1994
96-0658	3	1	1	3		Angola		1996
97-0483	3	1	1	3		Ghana		1997
BK04-0875	3	1	1	3		Togo	4+	2004
BK04-1396	3	1	1	3		Benin	–	2004
02-0280	3	1	1	3		Cameroon		2002
02-1081	3	1	1	3		Cameroon		2002
05-0303	3	1	1	3		Congo-Brazzaville		1979
05-0304	3	1	1	3		Congo-Brazzaville		1979
BK05-0027	3	1	1	3		Gabon	1+	2005
BK04-1591	4	1	1	1	East African Nile River Basin	Sudan	4+	2004
BK04-1601	4	1	1	1		Sudan	–	2004
05-0861	4	1	1	1		Orientale, DRC		1959
05-1459	4	1	1	1		Uganda (NCTC no. 10445)		1964
BK04-0513	4	1	1	1		Uganda	1+	2004
BK05-0614	4	1	1	1		Uganda	4+	2005

*MIRU, mycobacterial interspersed repetitive unit; VNTR, variable-number tandem repeat; PNG, Papua New Guinea; DRC, Democratic Republic of Congo; NA, no amplification; NCTC, National Collection of Type Cultures. Shaded fields represent results from our previous study (*6*).
†ITM numbers (Institute of Tropical Medicine). These numbers are representative members for the genotype each belongs to (*6*).
‡Numbers in columns 2 through 5 represent the number of repeats at the specific locus. These numbers form a pattern that divides *M. ulcerans* into genotypes.
§Scale of the American Thoracic Society. Ziehl-Neelsen staining has not been done on culture isolates, since identifying acid-fast bacilli in a culture is an obsolete practice.
¶The date represents the year of isolation.

confirmed cases of BU were decontaminated by using the reversed Petroff method, and mycobacterial DNA was extracted from the decontaminated solution as previously described (*6*). Smears of the suspensions were stained by the Ziehl-Neelsen method.

PCR was run as previously described (*6*). The Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany) was used to separate 1 μL of PCR product electrophoretically.

Comparison of MIRU-VNTR copy numbers using 4 loci showed 11 different profiles. *M. ulcerans* isolates from DRC and Uganda and tissue extracts from patients from Sudan (Nzara) and Uganda (Nakasongola) showed distinct profiles (Central Africa: 1222 and East Africa: 4111), different from the originally homogeneous African genotype (Atlantic Africa: 3113; Table 1). In DRC, 3 different genotypes exist, corresponding to 3 different provinces: Bas-Congo, Maniema (Kasongo), and Orientale (Bunia). The isolate from Orientale was from near the Ugandan border (Lake Albert). Isolates from Gabon, Congo-Brazzaville, and Cameroon had the typical African genotype, now designated the Atlantic African genotype. Identical MIRU-VNTR profiles were observed by using DNA extracted from tissues or cultures from patients residing in the same area. The specificity of the MIRU-VNTR method was tested on 14 different *Mycobacterium* spp. Only *M. marinum*, *M. shottsii*, and *M. liflandii* tested positive, but they were distinguished from *M. ulcerans* by exhibiting different profiles (data not shown). Sequencing of the concerned loci showed the conserved MIRU sequence at locus 1 and 9 in *M. ulcerans*. Locus 6 (3) and locus 33 contain respectively a 56-bp and a 58-bp tandem repeat (Table 2).

Table 2. Primer sequence and location in *Mycobacterium ulcerans* and amplicon length at loci 1, 6, 9, and 33, resulting from a polymorphism in tandem repeat copy numbers

Locus	Primer sequence		Location	Amplicon length			
	Forward primer (5′–3′)	Reverse primer (5′–3′)		1 copy	2 copies	3 copies	4 copies
1	GCTGGTTCATGCGTGGAAG	GCCCTCGGGAATGTGGTT	mu0115C04F	380	433	486	539
6	GACCGTCATGTCGTTCGATCC TAGT	GACATCGAAGAGGTGTGCC GTCT	mu0019B07G	500	556	–	–
9	GCCGAAGCCTTGTTGGACG	GGTTTCCCGCAGCATCTCG	mu0113D07F	435	488	–	–
33	CAAGACTCCCACCGACAGGC	CGGATCGGCACGGTTCA	mu043E11R	720	778	836	–

Conclusions

Although *M. ulcerans* isolates from Africa are relatively homogeneous, this study demonstrates more heterogeneity between strains than previously reported. All isolates from West Africa (Côte d'Ivoire, Ghana, Togo, Benin) and Central Africa (Cameroon; Gabon; Congo-Brazzaville; DRC Bas-Congo; Angola) have the identical MIRU-VNTR profile, and all originate from regions (i.e., Bas-Congo) or countries that border the Atlantic Ocean. The isolates that come from regions or countries in the Nile River basin (i.e., Orientale in DRC, Sudan, and Uganda) or the Congo River basin (i.e., Maniema) have distinct profiles.

These results demonstrate for the first time heterogeneity among *M. ulcerans* from different African countries. The 3 African profiles are the Atlantic African profile, the Central African Congo River basin profile, and the East African Nile River basin profile. This is also the first detection of MIRUs and VNTRs in clinical specimens, even in smear-negative specimens.

These data show that MIRUs and VNTRs are helpful tools in genotyping *M. ulcerans*. Further detailed differentiation of this etiologic agent will lead to an understanding of the epidemiology of BU. As in tuberculosis, better discriminatory typing methods help assess the efficacy of antimycobacterial treatment of BU patients by differentiating reactivation from reinfection. Although *M. ulcerans* appears to be quite monomorphic, full sequencing of this organism will permit detection of genes specific for *M. ulcerans*, and more discriminatory VNTR should become available.

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Mr Stragier is a doctoral student at the Mycobacteriology Unit, Institute of Tropical Medicine in Antwerp, Belgium. His research focuses on molecular microbiology and epidemiologic and environmental aspects of *M. ulcerans*. He is currently engaged in the Burulico project of the European Union to improve control of BU in Africa.

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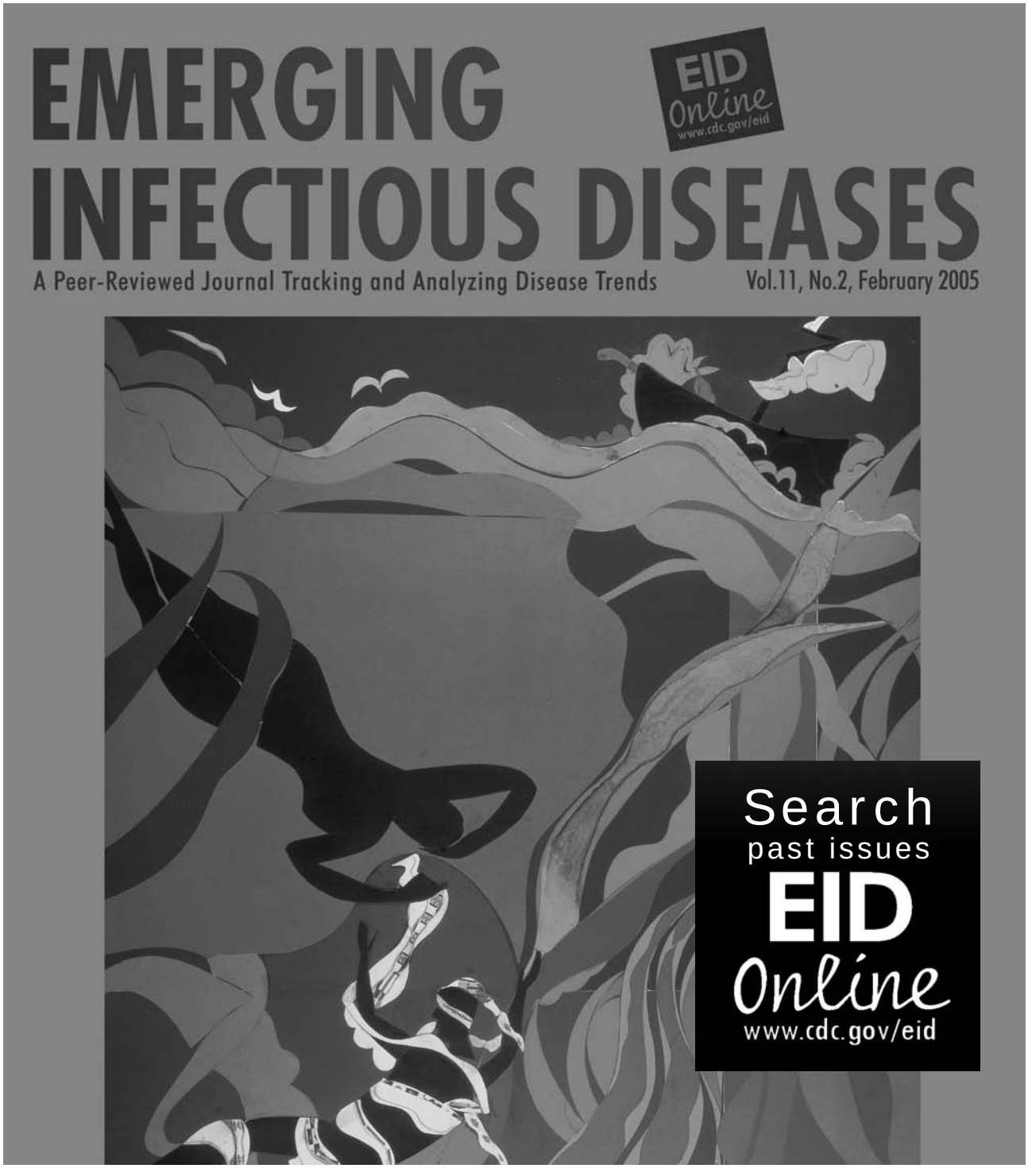

Human Bocavirus Infection, Canada

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Human Bocavirus was detected in 18 (1.5%) of 1,209 respiratory specimens collected in 2003 and 2004 in Canada. The main symptoms of affected patients were cough (78%), fever (67%), and sore throat (44%). Nine patients were hospitalized; of these, 8 (89%) were <5 years of age.

A new parvovirus, human Bocavirus (HBoV), was recently identified in Sweden (*1*). The virus was identified in clinical specimens from infants and children with respiratory tract illness. Phylogenetic analyses of the complete genome of HBoV showed that that the virus is most closely related to canine minute virus and bovine parvovirus, which are members of the genus *Bocavirus*, family *Parvoviridae* (*1*). To date, the only parvovirus known to be pathogenic in humans is B19, which is responsible for Fifth disease in children (*2*). The role of HBoV in respiratory tract illnesses is unknown. We retrospectively investigated HBoV in Canadian patients with acute respiratory infection (ARI) in 2003 and 2004 to assess the impact of HBoV infections on respiratory tract illnesses and identify the signs and symptoms of this illness.

The Study

A total of 1,209 specimens from patients with ARI from January 2003 to December 2004 were tested for HBoV. The specimens originated from the Saskatchewan provincial public health laboratories. Specimen types analyzed included throat swabs, nasopharyngeal swabs, nasopharyngeal aspirates, and auger suctions. All specimens were negative for influenza viruses A and B; parainfluenza viruses 1, 2, and 3; adenovirus; and respiratory syncytial virus (RSV) by direct or indirect fluorescence assays or virus isolation and for human metapneumovirus (HMPV) by reverse transcription–polymerase chain reaction. Specimens were collected from all age groups: 290 (24%) from those ≤5 years of age, 59 (5%) from those 6–10 years of age, 90 (7.4%) from those 11–15 years of age, 86 (7.1%) from those 16–20 years of age, 358 (29.6%) from those 21–50 years of age, and 324 (27%) from those >50 years of age, The age of the patients was unknown for 2 (0.2%) specimens.

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HBoV was detected by polymerase chain reaction (PCR) using primers specific for 2 different regions of the genome. The screening primers 188F (2281-5′-GACCTCTGTAAGTACTATTAC-3′-2301) and 542R (2634-5′-CTCTGTGTTGACTGAATACAG-3′-2614), reported by Allander et al. (*1*), were based on the sequence of the putative noncapsid protein 1 (NP-1) gene. The second set of primers, VP1/VP2F (4492-5′-GCAAACCCATCACTCTCAATGC-3′-4513) and VP1/VP2R (4895-5′-GCTCTCTCCTCCCAGTGACAT-3′-4875), was used for confirmation and was based on the published HBoV putative VP1/VP2 gene sequences (DQ000495) (*1*). Viral DNA was extracted from 285 μL of original samples with a BioRobot MDx and the QiAamp Virus BioRobot MDX kit (Qiagen, Valencia, CA, USA). We used 5 μL of DNA in a volume of 50 μL containing 20 pmol of each primer. The thermocycler conditions were 95°C for 15 min for activation of HotStartTaq DNA polymerase (Qiagen); 35 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and extension at 72°C for 10 min. Nucleotide sequences of NP-1 gene amplicons were determined with an ABI 377 Sequencer and a fluorescent dye terminator kit (Applied Biosystems, Foster City, CA, USA). DNA sequences were assembled and analyzed with SEQMAN, EDITSEQ, and MEGALIGN programs in Lasergene (DNASTAR, Madison, WI, USA). To avoid cross-contamination, specimen processing, DNA extraction, amplification, and analyses were conducted in different rooms. For DNA extraction and PCR procedures, we included 12 negative controls per 96-well plate.

A total of 18 (1.5%) of the 1,209 specimens tested were positive for HBoV by PCR. HBoV activity was found throughout the year with no apparent seasonal prevalence (Table 1). The sex distribution of patients was 61% (11) male and 39% (7) female (Table 2). Patients with HBoV ranged in age from 10 months to 60 years (median 11.5 years), and no significant difference in infection rates was observed between age groups.

The main clinical symptoms were cough (78%), fever (67%), and sore throat (44%) (Table 2). Other clinical symptoms included flulike symptoms (28%), headache (22%), nausea (17%), and myalgia (11%). Five patients had rhinitis, 1 had pneumonia, and 1 had bronchiolitis. One patient had rhinitis, bronchiolitis, and pneumonia, and 1 patient had rhinitis and pneumonia. Nine (50%) HBoV patients were hospitalized; 8 (89%) were ≤5 years of age, and 1 was between 21 and 50 years of age. The incidence of lower respiratory tract infection was lower in outpatients: 1 with bronchiolitis and no pneumonia (Table 2). Although the infection rates were similar in all age groups, a significant increase in hospitalization rates was seen in those ≤5 years of age compared with those >6 years of age (8/8 vs. 1/10, $p = 0.001$) (Table 2). All patients with

Table 1. Distribution of human Bocavirus–positive specimens by month, Canada

Date	No. positive/no. tested
2003	
Jan	1/48
Feb	2/51
Mar	0/44
Apr	1/49
May	0/48
Jun	4/54
Jul	1/53
Aug	0/46
Sep	0/49
Oct	0/51
Nov	1/50
Dec	1/50
2004	
Jan	0/50
Feb	1/51
Mar	0/48
Apr	0/50
May	0/50
Jun	0/50
Jul	1/50
Aug	0/66
Sep	1/52
Oct	0/49
Nov	3/50
Dec	1/50

pneumonia (3/3) and half of those with bronchiolitis (1/2) were also in this age group.

Nucleotide sequences were determined for nucleotides 2342–2581 that encode the NP-1 gene of HBoV (GenBank accession nos. DQ267760–DQ267775). No differences in nucleic acid sequences were found between different Canadian isolates. These isolates were also identical to 2 Swedish isolates (ST1 and ST2, GenBank accession nos. DQ000495–DQ000496) (*1*).

Conclusions

Although a causal relationship still needs to be demonstrated by including a control group of healthy persons, detection of HBoV in respiratory tract specimens from patients with undiagnosed ARI suggests that this virus may be associated with respiratory illness. This finding supports those of Allander et al. with regard to the association of HBoV with respiratory disease (*1*). It also demonstrates that HBoV was present in Canada in 2003 and 2004, which suggests that it may be circulating worldwide. Since this study used only samples from ARI patients who tested negative for influenza viruses A and B, parainfluenza viruses 1–3, adenovirus, RSV, and HMPV, dual infection cannot be excluded. In addition, whether HBoV is present asymptomatically in humans cannot be excluded because samples from healthy persons were not tested.

Allander et al. reported HBoV only in infants and children, which was probably the result of testing fewer specimens from adults patients (*1*). Most respiratory viruses show a seasonal distribution with peak activity in winter. Human parvovirus B19, the only parvovirus that is pathogenic in humans, is also seasonal, with peak occurrences in spring and summer (*3*). In contrast, no seasonal prevalence was observed for HBoV infection; the virus was found throughout the year. The lack of seasonality observed for HBoV may have been caused by the low prevalence in this study. Thus, additional year-round studies are needed to better understand the epidemiology of HBoV. Most (89%) hospitalizations were in persons ≤5 years of age, which

Table 2. Data from medical files of patients infected with human Bocavirus, Canada*

Specimen no.	Date collected	Sex	Patient status	Age	Symptoms
883	Jan 17, 2003	M	O	23 y	Fever, cough
947	Feb 5, 2003	M	H	9 mo	Fever, cough, nausea, rhinitis, pneumonia
963	Feb 28, 2003	F	O	11 y	Fever, cough, sore throat, rhinitis
1029	Apr 10, 2003	F	O	16 y	Sore throat, headache
1122	Jul 8, 2003	F	H	1 y	Fever, cough
1166	Jun 10, 2003	M	O	17 y	Sore throat, rhinitis
1178	Jun 16, 2003	M	H	28 y	Fever
1179	Jun 16, 2003	F	H	3 y	Fever, cough, rhinitis
1181	Jun 18, 2003	M	H	1 y	Fever, cough, rhinitis
1368	Nov 1, 2003	F	O	60 y	Cough, flulike symptoms, myalgia, headache, nausea
1431	Dec 16, 2003	F	O	41 y	Cough, sore throat, flulike symptoms, bronchiolitis
1545	Feb 24, 2004	M	H	10 mo	Fever, cough, pneumonia
1776	Jul 12, 2004	M	H	2 y	Fever
1871	Sep 3, 2004	M	H	11 mo	Cough, rhinitis, bronchiolitis, pneumonia
1979	Nov 8, 2004	F	O	12 y	Fever, cough, sore throat, flulike symptoms, headache
2013	Nov 28, 2004	M	H	9 mo	Cough
2016	Nov 25, 2004	F	O	14 y	Fever, cough, sore throat, flulike symptoms
2021	Dec 1, 2004	M	O	37 y	Fever, cough, sore throat, flulike symptoms, headache

*O, outpatient; H, hospitalized.

suggests that HBoV may cause more severe respiratory illness in infants and children, similar to disease caused by RSV (*4*,*5*), HMPV (*6*,*7*), human coronavirus NL63 (*8*–*14*), and human coronavirus 229E (*15*). More comprehensive studies with data on prevalence, risk factors, and use of health services are needed to determine the role of HBoV in ARI and its effect on the healthcare system.

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Lymphocytic Choriomeningitis in Michigan

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We summarize the first reported case of acquired lymphocytic choriomeningitis virus (LCMV) infection in Michigan to be investigated by public health authorities and provide evidence of the focal nature of LCMV infection in domestic rodents. Results of serologic and virologic testing in rodents contrasted, and negative serologic test results should be confirmed by tissue testing.

Lymphocytic choriomeningitis virus (LCMV) is a frequently unrecognized cause of aseptic meningitis and congenital infections in humans (*1*,*2*). First described in 1933, it is a rodentborne zoonosis associated with the common house mouse (*Mus musculus*) (*3*). Wild mice, often infected in utero, may not develop an effective immune response and remain asymptomatic carriers and shedders. Large-scale outbreaks of LCMV infection in humans have primarily been associated with contact with infected hamsters. Since 1960, 3 epidemics of LCMV infection involving at least 236 human cases have occurred in the United States; all were associated with Syrian hamsters as laboratory animals or pets (*4*). LCMV is shed in the urine, feces, saliva, milk, semen, and nasal secretions of chronically infected rodents. Routes of human exposure include aerosols, droplets, fomites, and direct contact with rodent excreta or blood (*3*). Recently, organ transplantation has been recognized as an additional mode of transmission for this virus (*5*). We describe the first reported case of meningitis due to LCMV infection in a Michigan resident.

The Case

A 46-year-old woman previously in good health came to a community hospital emergency department on June 12, 2004, with a 1-week history of severe headache, body aches, photophobia, weakness, and fatigue. A viral syndrome was diagnosed on 2 previous physician visits. Prior medical history included migraine headaches. A complete blood count, blood culture, serum chemistry tests, chest radiograph, urinalysis, and lumbar puncture were performed. Abnormal results included the following: cerebrospinal fluid contained 520 leukocytes/mm^3 with 100% lymphocytes, 19 erythrocytes/mm^3, protein 128.6 mg/dL, and glucose 59 mg/dL. Serum glucose was 124 mg/dL; serum lipase level was elevated at 686 U/L. A computed tomographic (CT) scan of the brain without infusion showed no evidence of acute brain process; abdominal/pelvic scan showed inflammatory change adjacent to the tail of the pancreas, consistent with possible pancreatitis.

The patient was admitted to the hospital and placed in respiratory isolation with a diagnosis of acute meningitis, likely of viral origin, and mild pancreatitis. She was given supportive care with intravenous fluids, acyclovir, and pain medication.

After consultation with an infectious disease specialist, several diagnostic tests were performed, including serologic tests for adenovirus, *Chlamydophila psittaci*, antinuclear antibodies, cytomegalovirus, LCMV, coxsackie B virus types 1–6, and echovirus types 4, 7, 9, and 11; polymerase chain reaction (PCR) for herpes simplex virus 1 and 2; infectious mononucleosis screen; cryptococcal antigen testing; and urinary mumps antibody testing. Positive results included mumps antibody titer of immunoglobulin G (IgG) 2.66 (negative <0.91) and IgM 1.64 (negative <0.81), and LCMV immunofluorescence assay (IFA) titer of IgG 256 (negative <15) and IgM 320 (negative <20). Confirmatory testing at the Centers for Disease Control and Prevention (CDC) in Atlanta found the specimen IgG-reactive and negative for mumps by urinary antigen culture and PCR. The patient was born before widespread mumps vaccination; thus, results suggested a previous exposure. LCMV serologic testing by enzyme-linked immunosorbent assay (ELISA) showed an IgG titer of 1,600 (cutoff <100) and an IgM titer of 6,400, which indicated recent infection.

The patient improved and was released after 7 days of hospitalization. The family had owned 2 healthy pet rats for 2 years, although the patient had little direct contact with them. However, the patient reported that the family had been battling a severe rodent infestation for 6 months, since they no longer kept cats as pets. The family had been trapping 4–5 mice per night in the weeks before the patient's illness onset. No other family members reported illness.

Because of the substantial rodent infestation and continuing risk to others in the household, the Michigan Department of Community Health, together with the local health department and the US Department of Agriculture

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Wildlife Services, received permission from the patient to conduct a field study to determine the extent of infestation and prevalence of infection in mice and to provide counseling on health implications and control of the infestation.

The Investigation

Following an initial site investigation, live traps were placed within the home and in the immediate area outdoors (within 10 m of the residence). Traps were visited daily for 2 days. All trapping and sampling procedures were performed according to CDC guidelines for sampling small mammals for virologic testing (*6*,*7*).

On July 28 and 29, 20 animals were captured, including 17 house mice, 1 white-footed mouse (*Peromyscus leucopus*), 1 short-tailed shrew (*Blarina brevicauda*), and 1 eastern chipmunk (*Tamias striatus*). Fecal pellets were also collected from the environment, traps, and pet rats' cage. From July 25 to 27, the homeowner caught 6 house mice in snap traps; the mice were frozen, and specimens were obtained.

During necropsy, blood samples were collected by saturation of Nobuto filter strips, and spleens were collected and frozen at −70°C. Spleen tissues and fecal pellets were homogenized, filtered, and inoculated into Vero cell cultures, which were maintained every 7 days with fresh maintenance media and observed daily for cytopathic effect. Serologic testing was performed on the Nobuto strips by CDC according to previously described methods (*8*). All cultures were screened by IFA staining with anti-LCMV mouse hyperimmune ascites fluid, obtained from CDC (lot #92-0038L) and diluted 1:800 in phosphate-buffered saline with 5% skim milk and 0.5% Tween 20 for 30 min at 37°C.

Twenty-two (96%) of 23 house mouse spleen tissue samples showed evidence of LCMV infection by virus isolation and IFA with specific LCMV antibodies. None of 14 fecal pellet suspensions showed evidence of LCMV by virus isolation or IFA. All Nobuto strips were negative for LCMV-specific antibodies by ELISA (Table). Confirmation PCR of a single virus isolate was conducted at the Special Pathogens Laboratories (CDC), and results were positive.

Five Vero cultures, positive by IFA, were observed by negative-stain electron microscopy grid preparation. This procedure confirmed virions consistent with an arenavirus in all specimens tested (Figure).

Conclusions

We describe the first documented case of acquired LCMV infection in Michigan. Evidence shows the highly focal nature of LCMV and the potential for human illness from exposure to the virus. Based on the patient's course of illness, dense rodent infestation in the patient's home, known routes of virus shedding, and mating and territorial ecology of the house mouse, we infer that the high infection rates in house mice caused her infection and subsequent illness. Investigators could not obtain samples from other residents of the house, so the household seroprevalence is undetermined.

Previous rodent serosurveys have shown focality, but few have provided evidence of such high infection rates in rodents. In this study, 96% of *M. musculus* examined were viremic. This result may be attributable to methods used to quantify infection status in the samples and the trapping intensity at a single focus. Infection rates of captured rodents may differ between rural and urban ecosystems, parks and housing complexes, and between housing complexes (*8*). Infection rates in natural populations have been estimated at 2.5% (California) and 21% (Washington, DC) (*9*,*10*). In urban Baltimore, however, single-dwelling units in the same neighborhood showed antibody prevalence to LCMV from 0% to 50% (*8*). In an LCMV epizootic of laboratory mice in the United Kingdom, bite transmission occurred and antibody prevalence was 67% in wild mice that were caught (*11*). Over a few generations, every member of a colony may become infected, as vertical transmission approaches 100% efficiency (*12*).

As was demonstrated by our results and suggested in earlier research, serologic testing of rodents underestimated overall infection rate (*8*), possibly because circulating antibodies were lacking in vertically infected mice. Oldstone and Dixon found that in the offspring of infected mice, antibodies to LCMV were sequestered in the kidneys and undetectable in blood (*13*). In our study, results of serologic testing on Nobuto strips were negative for all specimens, while results of virus isolation and IFA from spleen homogenates were positive for LCMV in 96% of *M. musculus* that were sampled and in 85% of all animals tested.

Table. Results of small mammal trapping and laboratory testing in a household exposure, July 25–29, 2004*

Species†	No. sampled	ELISA	Virus isolation‡ and IFA (%)	EM (%)
Mus musculus	23	0/23	22/23 (96)	5/5 (100)
Peromyscus leucopus	1	0/1	0/1	0/0
Tamias striatus	1	0/1	0/1	0/0
Blarina brevicauda	1	0/1	0/1	0/0

*LCMV, lymphocytic choriomeningitis virus; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; EM, electron microscopy.
†All species other than *M. musculus* were trapped outside the residence; 1 *M. musculus* was trapped outside, and the specimen was verified virus positive.
‡One isolate provided to the Centers for Disease Control and Prevention was confirmed LCMV-positive by polymerase chain reaction.

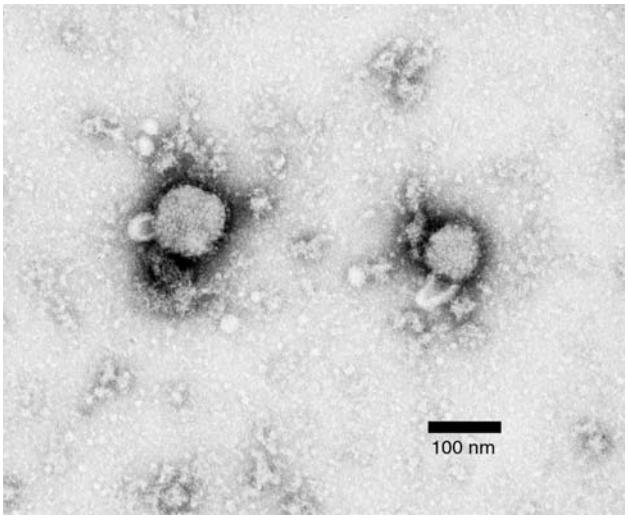

Figure. Methylamine tungstate negative-stain electron micrograph of arenavirus isolated from mouse spleen homogenate cultures that tested positive by immunofluorescence assay for lymphocytic choriomeningitis virus infection. Viral envelope spikes and projections are visible, and virion inclusions show a sandy appearance, indicating *Arenaviridae*.

Thus, negative serologic test results in rodents should be confirmed by tissue testing, such as virus isolation and IFA, or other methods, such as PCR. While most house mice were infected, none of the fecal pellets collected from traps were positive by virus isolation. This finding may be due to the fragile nature of LCMV in the environment or the predilection of the virus for rodent kidneys (*14*).

LCMV most likely represents an underdiagnosed, endemic zoonotic disease. Future goals include public health surveillance enhancements, physician education, and epidemiologic studies. Surveillance can be improved by adding LCMV to reportable disease lists and including a question about rodent exposure on case report forms for aseptic meningitis. Improved surveillance data can be used to educate clinicians on the range of illnesses caused by LCMV and the potential for acquired and congenital infection by exposure to rodents; this increased awareness would increase diagnostic testing and case identification. Improved case identification could lead to future studies to determine potential environmental, social, and economic risk factors, which would allow prevention and control efforts to be focused on vulnerable populations.

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Second Human Case of Cache Valley Virus Disease

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We document the second known case of Cache Valley virus disease in a human. Cache Valley virus disease is rarely diagnosed in North America, in part because laboratories rarely test for it. Its true incidence, effect on public health, and full clinical spectrum remain to be determined.

Cache Valley virus (CVV), a mosquitoborne member of the Bunyamwera serogroup, family *Bunyaviridae*, genus *Orthobunyavirus*, is geographically widespread in North America, where it circulates between mosquitoes and mammals (*1*). It has previously been associated with only a single case of human disease, a fatal case of acute encephalitis in the southeastern United States (*2*). We describe the second documented human case of CVV disease.

Case Report

In late October 2003, a 41-year-old, generally healthy Wisconsin man, who lived near a landfill in the suburbs of a small city on the Lake Michigan shore, became acutely ill with severe headache, nausea, vomiting, and fatigue. The next day, he was hospitalized with a diagnosis of acute aseptic meningitis. On admission, his body temperature was 38.4°C (101.1°F); no neck stiffness, rash, or focal neurologic abnormalities were detected. Computerized tomographic and magnetic resonance imaging scans of the brain were normal. The peripheral leukocyte count was 13,900/mm^3, with 87% neutrophils and 7% lymphocytes. Cerebrospinal fluid (CSF) examination showed 865 leukocytes/mm^3, with 73% lymphocytes, 15% monocytes, 12% neutrophils, and no erythrocytes; a protein concentration of 105 mg/dL, (normal 15–45 mg/dL); a glucose concentration of 47 mg/dL (normal 50–80 mg/dL); negative Gram stain; negative latex agglutination test results for antigens of *Neisseria meningitidis* groups A, B, C, Y, and W135, *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, *Escherichia coli* K1, and group B streptococci (Directigen Meningitis Combo Test Kit, BD, Franklin Lakes, NJ, USA); and negative routine bacterial cultures. Empiric intravenous antimicrobial drugs and corticosteroids were begun, and pain medications were administered. After 3 days, the patient's condition improved, and he was discharged on a tapering course of oral corticosteroids. Four months later, he reported feeling fully recovered except for experiencing headaches more frequently than usual.

After the patient's hospital discharge, the Wisconsin State Laboratory of Hygiene isolated a virus (designated strain WI-03BS7669) from an acute-phase CSF specimen. This isolate caused extensive cytopathic effects (CPE) in A549 (human lung adenocarcinoma) cells by 3 days after infection and in RD (human embryonal rhabdomyosarcoma) cells by 6 days, but no CPE were seen in primary monkey kidney or WI-38 (human embryonic lung) cells. Fluorescent-antibody test results of cell culture material were negative for adenoviruses, cytomegalovirus, varicella-zoster virus, herpes simplex virus, and enteroviruses, and polymerase chain reaction (PCR) assays for enteroviruses were negative. When electron microscopy of culture material showed virions morphologically similar to bunyaviruses, the isolate was sent to the Arboviral Diseases Branch of the Centers for Disease Control and Prevention (CDC) for characterization. By using primers targeted to a highly conserved 251-base portion of the smallest of the 3 RNA segments (RNA-S) of members of the Bunyamwera and California serogroups of the family *Bunyaviridae*, strain WI-03BS7669 was shown by PCR to share considerable homology with members of these serogroups (*3*). Subsequent nucleotide sequencing of ≈84% of RNA-S (795 of 950 total nucleotides in genome positions 84–878, GenBank accession no. DQ315775) followed by a BLAST (Basic Local Alignment Search Tool) search in GenBank showed that strain WI-03BS7669 was 99% identical to prototype CVV strain 6V633 (GenBank accession no. X73465; R.M. Elliott, pers. comm.) but only 90% identical to several other Bunyamwera serogroup viruses, including Potosi, Northway, Maguari, and Bunyamwera (*4*). In addition, a 694-base fragment amplified from the RNA-M segment of WI-03BS7669, followed by nucleic acid sequence analysis and a BLAST search, showed 98% sequence identity with 6 CVV strains but only 77% identity with Maguari virus (*4*).

No acute-phase serum was available for arboviral serologic testing. However, convalescent-phase serum collected from the patient 4 months after illness onset was strongly positive (titer 1,280) for neutralizing antibody to

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CVV strain 6V633 in 90% plaque-reduction tests conducted at CDC.

Conclusions

The Bunyamwera serogroup includes ≈30 viruses, with known representatives on every continent except Antarctica (R. Weir, pers. comm.) (*1*,*5*,*6*). Transmission cycles for these viruses have been little studied, but most isolates have been from culicine and anopheline mosquitoes, and mammals are considered the primary amplifying hosts. In Africa and Central and South America, Bunyamwera serogroup viruses cause sporadic human illness, including undifferentiated febrile illness, fever with exanthem, meningitis, and rarely, encephalitis. At least 7 different Bunyamwera serogroup viruses have been isolated from humans, usually from blood, including Bunyamwera, Germiston, Ilesha, and Shokwe viruses in Africa; Xingu virus in South America; Wyeomyia virus and a Cache Valley–like virus in Central America; and CVV in the southeastern United States (*1*,*2*,*5*,*7*,*8*). Ngari virus, a reassortant bunyavirus with RNA segments from both a Bunyamwera serogroup virus and an unidentified member of the genus *Orthobunyavirus*, has recently been associated with epidemic hemorrhagic fever in Africa (*9*).

CVV is 1 of at least 9 Bunyamwera serogroup viruses in North America (*1*,*5*,*6*,*10*), where it was first isolated from *Culiseta inornata* mosquitoes collected in Cache Valley, Utah, in 1956 (*5*). Although it has been isolated from >20 different species of culicine or anopheline mosquitoes, most frequently from *Anopheles quadrimaculatus* (*1*,*5*), the principal mosquito vectors are unknown. The vertebrate amplifying hosts of CVV have been little studied, but a high prevalance of neutralizing antibody to this virus is often found in ungulates, including deer, sheep, horses, and cattle (*5*,*10*–*12*). The virus has been isolated from a healthy cow and a sick sheep in Texas (*13*) and from a healthy horse in Michigan (*12*). It is teratogenic in sheep (*14*).

Previous serosurveys have indicated that humans in some parts of the United States are commonly infected by Bunyamwera serogroup viruses. For example, neutralizing antibody to CVV was found in 12% of 356 persons surveyed in Maryland and Virginia in the 1960s (*15*). Such results, however, are often difficult to interpret because of nonrandom sampling, multiple Bunyamwera serogroup members circulating in the same area, inclusion of a limited number of viruses in tests, and serologic cross-reactivity among members of the serogroup.

Only 2 cases of human disease due to Bunyamwera serogroup viruses were previously reported in temperate North America. The first was an encephalitis case in Indiana attributed to Tensaw virus in 1964 (*5*). Unfortunately, because no details about this case or the method of diagnosis were provided, and because the known range of Tensaw virus does not include Indiana, the validity of this report is uncertain. The second was a fatal, culture-confirmed case of CVV encephalitis in a young adult in North Carolina in 1995 (*2*).

Thus, our case of CVV meningitis is only the second documented human case of CVV disease. This case apparently lacked any unique clinical or routine laboratory features, and the diagnosis of CVV disease was made by the isolation of this virus from CSF by a state public health reference laboratory. The viral genomic sequence data and high-titer neutralizing antibody to CVV in the patient's convalescent-phase serum confirmed this case to be an acute CVV infection. Few CSF specimens are cultured for arboviruses because relatively few diagnostic laboratories have the expertise to do so and because even in acute, serologically confirmed cases of neuroinvasive arboviral disease, the isolation rate from CSF is generally low. No tests for CVV immunoglobulin M, such as enzyme immunoassay, are available. Tests for neutralizing antibody to this virus require handling live virus under biosafety level 2 containment and thus are only available by special request at CDC (through state health departments) and selected reference laboratories. In conclusion, CVV disease is a neuroinvasive illness rarely diagnosed in North America, in part because laboratories rarely test for it. Its true incidence, effect on public health, and full clinical spectrum remain to be determined.

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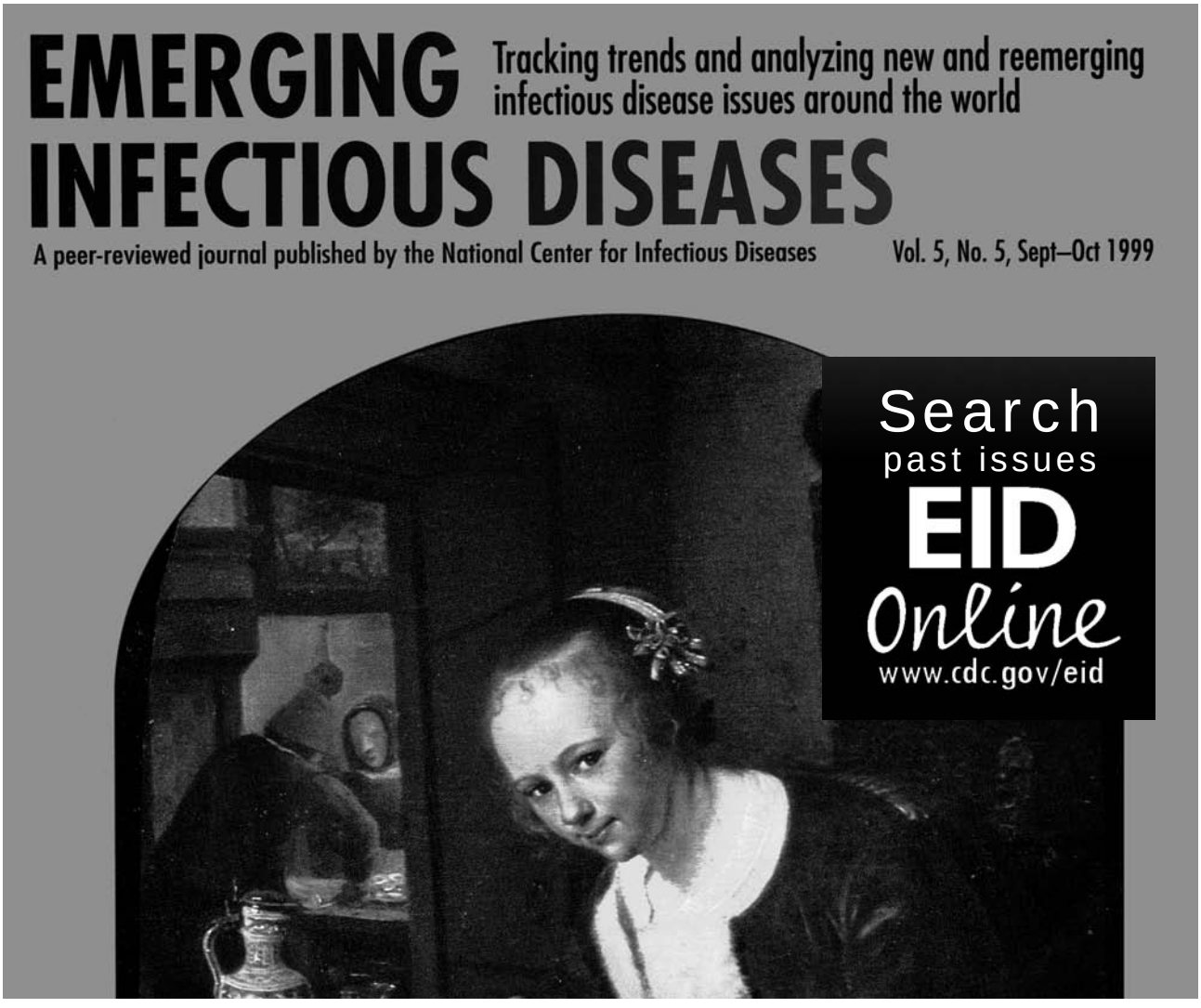

Novel Recombinant Norovirus in China

To the Editor: Norovirus (NoV), the distinct genus within the family *Caliciviridae*, is a major cause of sporadic cases and outbreaks of acute gastroenteritis in humans (*1*). NoV possesses a positive-sense, single-stranded RNA genome surrounded by an icosahedral capsid. The NoV genome contains 3 open reading frames (ORFs). ORF1 encodes nonstructural proteins, ORF 2 encodes capsid protein (VP1), and ORF3 encodes a small capsid protein (VP2). NoV is still uncultivable by standard culture with different cell lines. However, expression of either VP1 or both VP1 and VP2 with recombinant baculoviruses formed viruslike particles that are morphologically and antigenically similar to the native virion (*2*).

A fecal specimen was collected from an infant hospitalized with acute gastroenteritis in Kunming, China, in November 2004 and was tested for diarrheal viruses in a cooperative laboratory in Japan. The viral genome was extracted by using a Qiagen kit (Qiagen, Hilden, Germany). Polymerase chain reaction with specific primers resulted in the identification of astrovirus, rotavirus, sapovirus, adenovirus, and NoV genogroup I (GI) and GII (*3*). NoV polymerase was also amplified to identify recombinant NoV with primers Yuri22F and Yuri22R (*4*). Products were sequenced directly, and sequence analysis was performed by using ClustalX and SimPlot.

The fecal specimen was positive for NoV GII. The Figure shows that the 146/Kunming/04/China sequence clustered into the distinct GII genotype 7 (Leeds/90/UK cluster). 146/Kunming/04/China was classified into the Saitama U4 cluster (GI/6) when polymerase-based grouping was performed. Altogether, 146/Kunming/04/China was expected to be the recombinant NoV with GII/7 capsid and GII/6 polymerase.

To eliminate the possibility of coinfection with 2 different NoV genotypes, to localize the potential recombination site, and to clarify a possible recombination mechanism, the ORF1/ORF2 overlap and flanking polymerase and capsid regions of 146/Kunming/04/China was amplified with primers Yuri22F and GIISKR to produce a 1,158-bp amplicon (*3*,*4*). When the sequence of 146/Kunming/04/China was compared with that of Saitama U4 by using SimPlot, a recombination site was found at the ORF1/ORF2 overlap. Before this junction, 146/Kunming/04/China and Saitama U4 were homologous. After the ORF1/ORF2 overlap, however, the homology was notably different. SimPlot showed a sudden drop in the nucleotide identity for 146/Kunming/04/China. ClustalX showed that 146/Kunming/04/China shared a high identity (93%) in the polymerase region and a low identity (78%) in the capsid region with Saitama U4. In contrast, high identity (95%) in the capsid region was found between 146/Kunming/04/China and Leeds/90/UK. Since Leeds/90/UK polymerase was not available in GenBank, the polymerase homology between 146/Kunming/04/China and Leeds/90/UK was unknown. Polymerase of 146/Kunming/04/China was almost

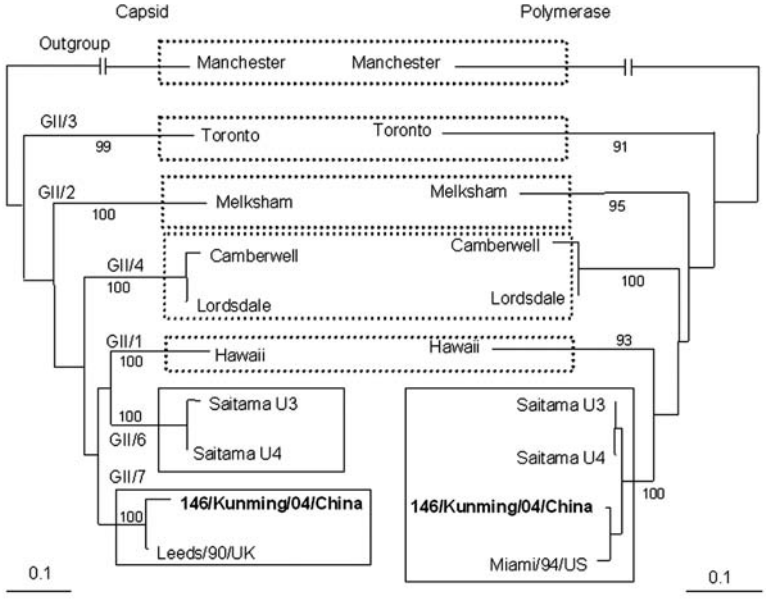

Figure. Changes in norovirus (NoV) genotypes on the basis of phylogentic trees of nucleotide sequences of 146/Kunming/04/China. Trees were constructed from partial nucleotide sequences of capsid and polymerase regions of 146/Kunming/04/China. 146/Kunming/04/China is **boldface**. Dashed boxes indicate the maintenance of genotypes of reference NoV strains, and solid boxes indicate the involvement of NoV genotypes with recombinant NoV 146/Kunming/04/China. A phylogenetic tree with 100 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using the Kimura 2-parameter method (PHYLIP). The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. Manchester strain was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence of NoV strain 146/Kunming/04/China had been submitted to GenBank and has been assigned accession no. DQ304651. Reference NoV strains and accession nos. used in this study are as follows: Manchester (X86560), Toronto (U02030), Melksham (X81879), Camberwell (AF145896), Leeds/90/UK (AJ277608), Lordsdale (X86557), Hawaii (U07611), Saitama U3 (AB039776), Saitama U4 (AB039777), and Miami/94/US (AF414410).

identical with that of Saitama U4, but the capsids of 146/Kunming/04/China and Leeds/90/UK were distinctly different from that of Saitama U4. This genetic pattern of 146/Kunming/04/China implied a novel, naturally occurring recombinant NoV with GII/7 capsid and GII/6 polymerase.

RNA recombination is a mechanism for virus evolution (*5*). Literature documenting recombination in NoV is fairly rich, but none is from China (*6*). Therefore, 146/ Kunming/04/China was not only the first but also the first recombinant NoV from China. This isolate shared the closest sequences of polymerase and capsid with Saitama U4 and Leeds/90/UK, respectively. Strain Saitama U4 was detected in 1997 in Japan (*7*), whereas strain Leeds/90/ UK was detected in 1990 in the United Kingdom (*8*). Quite possibly, Saitama U4 and Leeds/90/UK were parental strains of 146/Kunming/04/China. However, the distant geographic relationship of these strains obscured evidence of where and when the recombination event occurred. This phenomenon also suggested that these parent strains or this progeny strain might be more prevalent than is often assumed.

Recombination depends on various immunologic and intracellular constraints. Recombinant viruses are all alike in that they successfully pass through 5 stages: 1) successful co-infection of a single host, 2) successful co-infection of a single cell, 3) efficient replication of both parental strains, 4) template switching, and 5) purifying selection (*9*). In this study, 146/Kunming/04/China was recovered from a patient with diarrhea, fever, and vomiting. This observation indicated that this strain theoretically fulfilled all prerequisites for recombination.

The NoV capsid is predicted to be well suited for genotype classification (*10*). In this study, 146/Kunming/04/China belonged to 2 distinct genotypes, 7 and 6, by capsid- and polymerase-based groupings, respectively. Moreover, the recent demonstration of recombination in an increasing number of NoVs suggests that it is a more widespread event than was previously realized. Consequently, the phylogenetic classification of NoV on the basis of on capsid sequence is questionable. We suggest that classification of NoV strains should rely on not only capsid sequence but also polymerase sequence.

In conclusion, our results described the genetic characterization of novel, naturally occurring recombinant NoV and increased evidence for the worldwide distribution of recombinant NoV. This report is the first to describe acute gastroenteritis caused by recombinant NoV in China and warns of the threat it poses.

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Instructions for Emerging Infectious Diseases Authors

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Rifampin-resistant *Neisseria meningitidis*

To The Editor: Immediate management of meningococcal disease requires antimicrobial drug treatment of patients with β-lactams and chemoprophylaxis of contact persons with rifampin. High-level resistance to rifampin (MIC >32 mg/L) in *Neisseria meningitidis* is provoked by mutations (most frequently at the residue His 552) in the *rpoB* gene encoding the β subunit of RNA polymerase (*1*,*2*). Resistance may lead to chemoprophylaxis failure and must be rapidly detected (*3*). Concerns have been raised about the clonal spread of resistant isolates (*1*); however, rifampin-resistant isolates are rarely reported. We tested 6 *N. meningitidis* isolates corresponding to 3 pairs of linked cases of meningococcal disease. In each pair, the index case was due to a rifampin-susceptible isolate and was followed by the secondary case due to a resistant isolate in a contact person. Phenotyping and genotyping of the isolates showed that each pair belonged to a different major serogroup (A, B, and C) and to a different genetic lineage (ST-7, ST-32, and ST-2794) (Figure). We next amplified a fragment in *rpoB* between codons 421 and 701 by using oligonucleotide rpoBF1 (5′gttttcccagtcacgacgttgta-CTGTCCGAAGCCCAACAAAACTCTTGG3′) and rpoBR1 (5′ttgtgagcggataacaatttcTTCCAAGAATGGAATCAGGGATGCTGC3′). The 2 oligonucleotides harbor adaptors (in lower case) corresponding to universal forward and reverse oligonucleotides that can be used for sequencing after amplification. We also analyzed 2 cerebrospinal fluid (CSF) samples corresponding to 2 linked culture-negative cases of meningococcal disease in which the second case was believed to have been caused by rifampin-resistant *N. meningitidis*. These 2 cases were diagnosed by polymerase chain reaction (PCR) detection of meningococcal DNA, as previously described (*4*).

The 3 rifampin-susceptible isolates harbored a wild-type *rpoB* sequence (His 552), as did the first CSF sample. All 3 rifampin-resistant isolates harbored a His→Tyr mutation, while analysis of the second CSF sample showed a His→Asn mutation (Figure). Both mutations have been observed in *N. meningitidis* (*3*). No other difference in the sequence was seen among all isolates on the amplified fragment. This approach can rapidly detect *rpoB* mutations and can be applied to culture-negative clinical samples.

The virulence of the isolates was evaluated through their ability to provoke bacteremia in mice after 6-week-old female BALB/c mice (Janvier, France) were injected intraperitoneally. Bacteremia is a good indicator of bacterial virulence as it reflects bacterial survival upon invasion of the bloodstream. The experimental design was approved by the Institut Pasteur Review Board. The rifampin-resistant clinical isolate LNP22330 showed substantially reduced bacteremia when compared to the corresponding susceptible isolate LNP21362 (Figure). Such a reduction was not significant for the other 2 pairs (LNP18278/LNP18378 and LNP18368/LNP18491), but these strains were all less virulent than LNP21362, with ≈1 $\log_{10}$ lower blood bacterial loads. The 3 pairs of isolates belonged to different genetic lineages according to the multilocus sequence typing typing. Indeed, we have recently proved that virulence of meningococcal isolates in the mouse model depends on the genetic lineage of the tested isolate (*5*).

To better study the impact of *rpoB* mutation on meningococcal virulence we constructed an isogenic mutant strain, NM05-08, by transforming the susceptible isolate LNP21362 with a PCR-amplified fragment from a resistant isolate (LNP22330), as previously described (*6*). The PCR fragment corresponded to the product of amplification between the oligonucleotides ropB1UP (5′ggccgtctgaaCTGTCCGAAGCCCAACAAAACTCTTGG3′) and rpoBR1. The oligonucleotide RpoB1UP is the same as

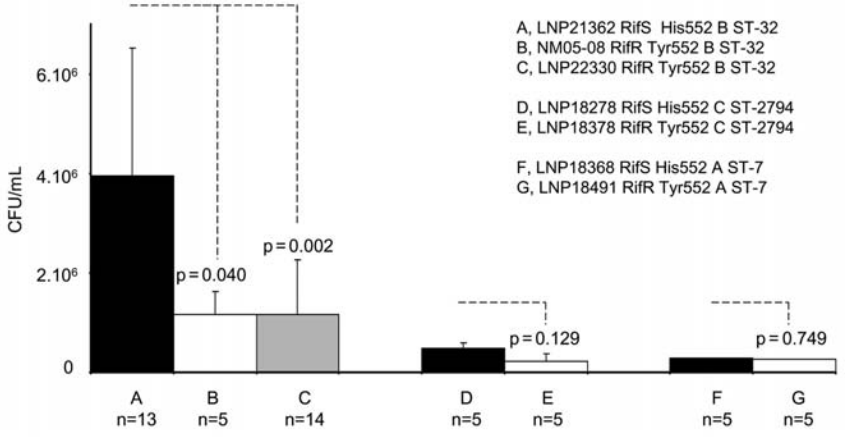

Figure. Blood bacterial counts in 6-week-old female BALB/c mice (Janvier, France), challenged intraperitoneally with standardized inocula of 10^7 colony forming units (CFU) of rifampin-susceptible (RifS) isolates and their corresponding rifampin-resistant (RifR) isolates. Bacteremia was followed at 2 and 4 h after challenge. Only results after 4 h of challenge are shown. The name of the isolates tested, their phenotype (susceptibility to rifampin, RifS or RifR, residue at the position 552 and serogroups B, C, and A), and their genotype (sequence type ST) are indicated. Results are the means ± standard error (bars) from groups of at least 5 mice (the number of mice, n, is given above each histogram). p values were determined by 2-tailed Student *t* test.

the upstream rpoBF1 but with a DNA uptake sequence (in lower case) that was added at the 5′ end to permit DNA transformation (*7*). The transformant strain NM05-08 was resistant to rifampin (MIC >32 mg/L), and the sequence of the *rpoB* gene confirmed the His→Tyr mutation. When compared to the parental isolate (LNP21362), strain NM05-08 showed reduced virulence. Indeed, bacterial loads were similar to those observed for the resistant isolate LNP22330 (Figure). These results strongly suggest a direct negative impact of *rpoB* mutations on meningococcal virulence. Mutations in the *rpoB* gene have been reported to confer pleiotropic phenotypes (*8*).

The data reported here show that rifampin-resistant isolates were not clonal but belonged to different genetic lineages. The results of virulence assays in mice suggest that mutations in *rpoB* in resistant isolates may have a major biological cost for *N. meningitidis*, which can be defined as lower bacterial fitness in terms of survival in the bloodstream. This biological cost could explain the lack of clonal expansion of meningococcal isolates that acquired resistance to rifampin.

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Vaccination-related *Mycobacterium bovis* BCG Infection

To the Editor: The high prevalence of tuberculosis (TB) underlines the important role of BCG (bacillus Calmette-Guérin) immunization. The vaccine, however, is not free from complications, which could be local or disseminated. Disseminated BCG infection as a result of TB vaccination is a rare complication with an incidence of 0.06 to 1.56 cases per million vaccinations; it occurs exclusively in patients with immune deficits. However, in these cases, the prognosis is unfavorable; up to 70% of patients die, despite intensive antituberculous treatment (*1–4*).

A 4-month-old-girl exhibited enlargement of left axillary lymph nodes during a 1.5-month period. She was the second child of healthy parents, with no family history of genetic disorders or TB. She was vaccinated according to the regimen compulsory in Poland: the first dose of BCG and anti–hepatitis B virus (HBV) vaccination on the first day of life, followed by vaccination against diphtheria, tetanus, pertussis, poliomyelitis, and the second dose of anti-HBV after 6 weeks. BCG vaccination was performed intradermally in the upper part of left arm by administration of 0.1 mL Brazilian Moreau strain (Biomed, Lublin, Poland).

On hospital admission, the patient was in reasonably good condition but pale, with grossly enlarged, adjacent left axillary lymph nodes and hepatosplenomegaly. Laboratory tests showed anemia, thrombocytopenia, elevated transaminase activity, a high C-reactive protein level, and high level of immunoglobulin M (IgM) class anti-cytomegalovirus (CMV) reactive antibodies.

Based on clinical manifestations and biochemical and serologic signs, CMV infection was suspected. The patient was administered a 14-day regimen of ganciclovir (10 mg/kg/day); results of liver function tests and blood count normalized, and hepatosplenomegaly decreased. However, the lymph nodes continued to enlarge, and diagnostic excision and bone marrow aspiration were performed to exclude a neoplastic process. A histopathologic image of the excised lymph nodes showed caseating granulomas, and tuberculous lymphadenitis was suggested (Figure).

At that time, a diagnosis of disseminated BCG infection as a complication of TB vaccination in a presumed immunocompromised patient

was proposed. This idea was based on suggestive lymph node pathology, which showed caseating granulomas, a history of TB vaccination, and the exclusion of other pathologic changes. Flow cytometry measurements showed abnormally low expression of the α chain of the interferon (IFN)-γ receptor on peripheral blood lymphocytes. Only 20% lymphocytes expressed CD 119 (IFN-γ receptor outer subunit R1).

Three-drug anti-tuberculous therapy (with rifampin, isoniazid, and streptomycin) was introduced despite chest and bone radiographs that were negative for infection, no abnormalities found on funduscopy, and negative results of Ziehl-Neelsen staining of lymph node tissue. Despite this therapy, the child's condition worsened; she exhibited a high temperature, hemolysis, and progressive neutropenia, thrombocytopenia, cholestasis, and renal failure. Uncontrolled sepsis developed, and she died.

At postmortem examination, the diagnosis of disseminated BGC infection was made on the basis of multiple TB-like granulomas in the lungs, lymph nodes, meninges, liver, spleen, and kidneys. However, direct microbiologic confirmation of BCG infection was lacking because cultures were negative and Ziehl-Neelsen and periodic acid–Schiff staining did not show acid-fast bacilli, other bacteria, or fungi in these specimens.

This case represents a rare complication of antituberculous vaccination, that is a progressive, disseminated BCG infection in a patient with deficiency of IFN-γ receptor. Concomitant CMV infection was diagnosed by positive IgM antibody response. Transient response to the ganciclovir treatment made the final diagnosis of BCG infection more difficult and probably postponed implementation of the anti-TB therapy. Until now ≈100 cases have been reported in the literature, most of them in infants and young children. These patients also had clear predisposition to other severe infections with intracellular microorganisms such as atypical mycobacteria, *Salmonella* spp., *Listeria monocytogenes*, and *Leishmania* spp. (*1–5*).

The INF-γ receptor is present on many cell types; however, its deficiency on macrophages may be responsible for the inhibition of phagocytosis and intracellular killing and the observed deficit of an antimycobacterial immunity. Among children with a clinical syndrome of IFN-γ–receptor deficiency, a clear genetic defect was identified in ≈20%. In our patient, the diagnosis was made by detection by flow cytometry of abnormally low expression of the α chain of the IFN-γ receptor on peripheral blood lymphocytes. This method appears to have high diagnostic value, given the fact that genetic methods are not always available and are expensive and often insensitive.

The prognosis in patients with BCG infection secondary to IFN-γ–receptor deficiency is unfavorable. A few cases of successful treatment with allogenic bone marrow transplantation have been reported with long-term improvement of general condition and stable receipt of the graft as shown by molecular analysis of peripheral leukocytes (*4*,*6–8*). However, as specific and efficient therapy for this condition has not been as yet proposed, supportive measures with early diagnosis and institution of anti-TB and antimicrobial drug treatment appear to be important in managing this rare immune deficiency. The level of IFN-γ–receptor expression in populations known to be susceptible to TB, and its potential role in this phenomenon, appears to be a promising area of study.

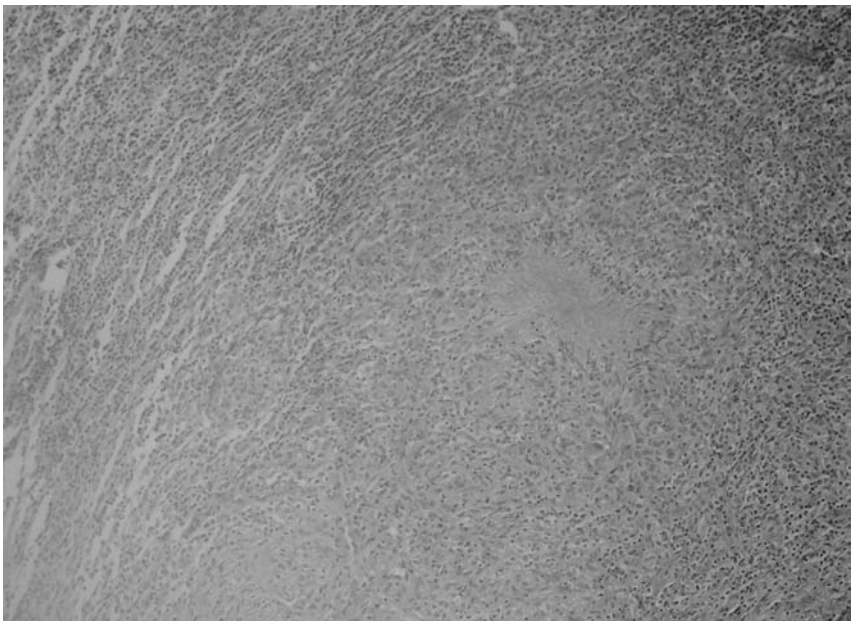

Figure. Digitally processed hematoxylin-eosin staining of the excised lymph nodes, showing caseating, tuberculosislike granulomas (original magnification ×100).

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Human Bocavirus in Children

To the Editor: Respiratory tract infection is a major cause of illness in children. Despite the availability of sensitive diagnostic methods, detecting infectious agents is difficult in a substantial proportion of respiratory samples from children with respiratory tract disease (*1*). This fact suggests the existence of currently unknown respiratory pathogens.

A new virus has been recently identified in respiratory samples from children with lower respiratory tract disease in Sweden (*2*). Analysis of the full-length genome sequence showed that this virus is closely related to bovine parvovirus and canine minute virus and is a member of the genus *Bocavirus*, subfamily *Parvovirinae*, family *Parvoviridae*. This virus has been provisionally named human Bocavirus (HBoV) (*2*). HBoV in respiratory samples from Australian children was also recently reported (*3*). Involvement of this new virus in respiratory tract diseases merits further investigation. We have therefore retrospectively tested for HBoV nasopharyngeal samples collected from children <5 years of age hospitalized with respiratory tract disease.

Samples were collected from 262 children from November 1, 2003, to January 31, 2004. The samples were tested for respiratory viruses by using direct immunofluorescence assays with monoclonal antibodies to respiratory syncytial virus; influenza virus types A and B; parainfluenza virus types 1, 2, and 3; and adenovirus. Samples were also placed on MRC5 cell monolayers for virus isolation and tested for human metapneumovirus by reverse transcription–polymerase chain reaction (RT-PCR). Nucleic acids were extracted from samples, stored at –80°C, and tested for HBoV DNA by PCR with primers specific for the predicted NP1 gene as previously described (*2*). The expected product size was 354 bp. In each experiment, a negative control was included, and positive samples were confirmed by analyzing a second sample. Amplification specificity was verified by sequencing.

Nine (3.4%) samples were positive. Comparison of PCR product sequences of these 9 isolates (GenBank accession nos. AM109958–AM109966) showed minor differences that occurred at 1 to 4 nucleotide positions, and a high level of sequence identity (99%–100%) was observed with the NP1 sequences of the previously identified ST1 and ST2 isolates (*2*). This finding indicates that HBoV is a highly conserved virus.

HBoV was the only virus identified in 6 children and was associated with respiratory syncytial virus in 3 other children. An infection with other respiratory viruses was detected among 153 (60.5%) of the 253 HBoV-negative children. The viruses identified were respiratory syncytial virus in 114 (43.5%) samples, human metapneumovirus in 27 (10.3%) samples, influenza A virus in 14 (5.4%) samples, rhinovirus in 4 (1.5%) samples, adenovirus in 2 (0.8%) samples, and parainfluenza virus type 3 in 1 (0.4%) sample. Respiratory syncytial virus was associated with human metapneumovirus in 9 (3.4%) samples.

Clinical characteristics of the HBoV-infected children are shown in the Table. Children infected with only HBoV had mild-to-moderate fevers. Leukocyte counts and C-reactive protein levels were normal or moderately elevated. Chest radiographs obtained for 7 children showed abnormalities such as hyperinflation and interstitial infiltrates. Bronchiolitis was the major diagnosis. Dyspnea, respiratory distress, and cough were the most common respiratory symptoms observed. Four (44%) HBoV-infected children were born preterm, which suggests that these children have an increased susceptibility to HBoV-associated diseases. All children

Table. Clinical characteristics of children infected with human Bocavirus*

Age (mo)	Sex	Copathogen	Fever (°C)	Leukocytes ($\times 10^3/\mu L$)	CRP (mg/L)	SaO_2 (%)	Underlying condition (wks of pregnancy)	Diagnosis	Symptoms†
8	M	RSV	39.0	NA	NA	NA	None	Bronchiolitis	D, C
39	M	RSV	38.5	14.6	13.0	95	Preterm (36)	Asthma	RD, D
12	F	RSV	37.5	15.9	13.6	NA	None	Bronchiolitis	RD, C, O
19	F	None	37.3	15.6	<5.0	91	Preterm (35)	Bronchiolitis	RD
8	M	None	36.8	NA	NA	95	None	Bronchiolitis	D
10	M	None	38.2	12.6	9.6	NA	Preterm (28)	Bronchiolitis	D
9	F	None	38.5	12.7	<5.0	68	Chronic respiratory disease	Acute respiratory distress	RD
14	M	None	38.1	9.0	38.5	93	None	Bronchiolitis	RD, D, C
11	M	None	37.8	9.4	<5.0	96	Preterm (31)	Asthma	D

*CRP, C-reactive protein; SaO_2 saturation of arterial oxygen; RSV, respiratory syncytial virus; NA, not available.
†D, dyspnea; C, cough; RD, respiratory distress; O, otitis.

recovered and were discharged within 1 to 6 days.

The 3.4% incidence of HBoV observed in our study is similar to that (3.1%) reported by Allander et al. (*2*). HBoV was the only infectious agent identified in 6 children, which suggests that it was the causative agent of the disease. However, more studies conducted in children with and without respiratory disease as well as in adults and elderly persons are needed to better assess the pathogenic role of HBoV.

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Extended-spectrum β-Lactamase–producing *Enterobacteriaceae*, Central African Republic

To the Editor: Since the early 1980s, extended-spectrum β-lactamases (ESBLs) have been the largest source of resistance to broad-spectrum oxyimino-cephalosporins among *Enterobacteriaceae* (*1*). Molecular analysis techniques suggest that many ESBLs are derived from mutations in TEM-1, TEM-2, and SHV-1 β-lactamases and that these ESBLs can hydrolyze the extended-spectrum cephalosporins (particularly ceftazidime) and aztreonam (*1*). Members of a new group of ESBLs have been recently identified (*1*). Among them, CTX-M–type ESBLs are rapidly expanding and are derived from chromosomal class A β-lactamases of *Kluyvera* spp. (*1*,*2*). The CTX-M enzymes are not related to TEM or SHV enzymes, as they share only 40% identity with these ESBLs (*2*).These ESBLS are usually characterized by a higher level of resistance to cefotaxime than ceftazidime, except for CTX-M-19 (*2*). Most organisms that harbor ESBLs are also resistant to other classes of antimicrobial drugs, such as aminoglycosides, fluoroquinolones, chloramphenicol, and tetracyclines (*1*,*2*).

Reports concerning the existence of ESBL-producing *Enterobacteriaceae* in sub-Saharan Africa are scarce. We therefore conducted a study in the Central African Republic to determine the frequency of ESBLs in *Enterobacteriaceae* isolated at the Institut Pasteur de Bangui and to characterize their bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes.

From January 2003 to March 2005, all *Enterobacteriaceae* isolated from human specimens at the Institut Pasteur de Bangui were screened for ESBLs. Antimicrobial drug susceptibility was determined by using the disk diffusion method (Bio-Rad, Marnes la Coquette, France) on Mueller-Hinton agar (MHA) and interpreted according to the recommendations of the Comité de l'Antibiogramme de la Société

Table. Characteristics of extended-spectrum β-lactamase–producing *Enterobacteriaceae* in Bangui, Central African Republic

Strain†	Patient hospitalized	Results of sequencing			MICs of β-lactams (μg/mL)*							Resistance
		bla_{CTX-M}	bla_{SHV}	bla_{TEM}	AMC	CTX	CAZ	CRO	FEP	CPO	ATM	
K. pneumoniae 022	N		SHV-2a	TEM-1	16	16	16	16	8	8	2	KGT
K. pneumoniae 043	Y		SHV-12	TEM-1	16	16	256	32	8	8	256	KGTNC
K. pneumoniae 106	Y	CTX-M-15		TEM-1	8	256	128	256	64	256	64	None
K. pneumoniae 047	Y		SHV-2a	TEM-1	64	16	16	16	8	16	32	None
E. coli 272	Y	CTX-M-15		TEM-1	32	256	128	256	128	256	128	KGTNC
E. coli 065	Y	CTX-M-15		TEM-1	20	256	128	256	64	256	128	C
E. coli 047	N	CTX-M-15		TEM-1	16	256	32	256	32	128	64	KGTC
E. coli 010	N	CTX-M-15		TEM-1	32	256	128	256	128	256	256	KGT
E. coli 073	N	CTX-M-15		TEM-1	16	256	128	256	128	256	128	KGTC
E. coli 059	Y	CTX-M-15		TEM-1	19	256	128	256	8	256	256	C
E. coli 064	N	CTX-M-15		TEM-1	128	256	128	256	64	256	64	C
E. coli 070	N	CTX-M-15		TEM-1	128	256	128	256	64	256	128	C
E. coli 054	N	CTX-M-15		TEM-1	128	256	32	256	64	256	32	KGTC
E. coli 026	N	CTX-M-15		TEM-1	32	256	64	256	128	256	256	KGTC
E. cloacae 081	Y		SHV-12	TEM-1	32	16	256	16	0.125	1	256	KGTN
E. cloacae 106	Y		SHV-12	TEM-1	128	16	256	16	32	8	256	KGT
E. aerogenes 014	Y	CTX-M-3	SHV-12	TEM-1	128	256	256	256	32	256	128	KGTN

*AMC, amoxicillin + clavulanic acid (2 μg/mL); CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; CPO, cefpirome; ATM, aztreonam; K, kanamycin; G, gentamicin; T, tobramycin; N, netilmicin; C, ciprofloxacin.
†On *Klebsiella pneumoniae* strains, polymerase chain reaction and sequencing for bla_{SHV} genes were studied on *Escherichia coli* transconjugant or electroporant.

Française de Microbiologie (CA-SFM) (www.sfm.asso.fr). ESBL-producing *Enterobacteriaceae* were selected by the following criteria: susceptibility to cefoxitin; decreased susceptibility to cefotaxime (30 μg), ceftazidime (30 μg), or cefepime (30 μg) (zone diameter <21 mm); and enhanced susceptibility in the presence of clavulanic acid by the double disk synergy test (*3*). For suspected ESBLs, the MICs of broad-spectrum cephalosporins were determined by using the agar dilution method.

We screened 450 *Enterobacteriaceae* for ESBLs during the study. We isolated and identified 17 (4%) ESBL-producing strains (Table). These strains were associated with urinary tract infection, pneumonia in an AIDS patient, wound infection, vaginal or intestinal colonization, and ear infection. We found that 11 isolates were more resistant to cefotaxime (MIC ≥256 μg/mL) than to ceftazidime (MIC ≤128 μg/mL), which suggests CTX-M–type enzymes. *Enterobacteriaceae* strains that harbor ESBLs were frequently associated with resistance to aminoglycosides and ciprofloxacin (Table).

The conjugal transfer of the resistance determinants was carried out in trypticase soy (TS) broth with rifampin-resistant *Escherichia coli* J53-2 as the recipient. Mating broths were incubated at 37°C for 18 h. Transconjugants were selected on MHA plates containing rifampin (250 μg/mL) and cefotaxime (2.5 μg/mL). If conjugal transfer failed, plasmid DNA was extracted from donors with the Qiagen Plasmid Mini Kit (Qiagen, Courtaboeuf, France); 20 μL of *E. coli* DH10B cells were transformed with plasmid DNA by electroporation according to the manufacturer's instructions (Bio-Rad). Transformants were incubated for 1.5 h at 37°C in TS broth and then plated on MHA plates supplemented with 2.5 μg/mL cefotaxime.

Plasmid-encoded β-lactamase genes were detected on clinical isolates and their tranconjugants or transformants by polymerase chain reaction with oligonucleotide primer sets specific for the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes (*4*). PCR assays were performed on total DNA extracted by using the commercial Qiagen DNA Mini Kit. The 3 β-lactamase genes were detected in different clinical isolates (Table). PCR results showed that the strains were harboring ≥2 different types of β-lactamases.

Plasmid-encoded β-lactamase genes were characterized by direct DNA sequencing with PCR primers. The nucleotide sequences were analyzed by the BLASTN (nucleotide basic local alignment search tool) program. For ESBLs, the gene types (SHV-2a, SHV-12, CTX-M-15, and CTX-M-3) were identified from different *Enterobacteriaceae* (Table). Only 1 strain (*Enterobacter aerogenes*) harbored 2 different ESBLs (CTX-M-3 and SHV-12). We identified TEM-1 and CTX-M15 enzymes, which are the most prevalent β-lactamases detected in our strains.

ESBL-producing *Enterobacteriaceae* have been previously described in South Africa (*5*), Kenya (*6*), Senegal (*7*), Cameroon (*8*), Tanzania (*9*), and Nigeria (*10*). As described in these countries, we found that CTX-M-15, SHV-2a, and SHV-12 were the most prevalent enzymes. CTX-M-15, the most recently described ESBL type, is particularly common in Bangui and seems to be

closely related to *E. coli*, as was previously observed in Tanzania (*9*). This finding is also the first report of CTX-M-3 in sub-Saharan Africa.

Multidrug resistance profiles involving non–β-lactam antimicrobial drugs coselected these ESBL-producing isolates. We suggest that the misuse of antimicrobial drugs in the Central African Republic and the migratory flux of regional populations could result in emergence and selection of these ESBL phenotypes in the community. We could not establish a relationship between the different strains isolated in hospitalized and ambulatory patients. Because of the implications for treating such infections, particularly in developing countries, the spread of ESBL-producing *Enterobacteriaceae* merits close surveillance in the Central African Republic.

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Novel Recombinant Sapovirus, Japan

To the Editor: *Sapovirus* is the distinct genus within the family *Caliciviridae*; these viruses cause sporadic cases and outbreaks of gastroenteritis in humans worldwide (*1*). The sapovirus genome contains 2 open reading frames (ORFs). ORF1 encodes nonstructural and capsid proteins while ORF2 encodes a small protein (*2*). Sapovirus has a typical "Star of David" configuration by electron microscopic examination. The prototype sapovirus is the Sapporo virus (Hu/SaV/Sapporo virus/1977/ JP), which was originally discovered from an outbreak in a home for infants in Sapporo, Japan, in 1977 (*3*). Sapovirus is divided into 5 genogroups, among which only genogroups I, II, IV, and V are known to infect humans (*4*).

A fecal specimen was collected from a 1-year-old boy with acute gastroenteritis in Osaka, Japan, in March 2005. The viral genome was extracted by using a QIAamp kit (Quigen, Hilden, Germany). By using multiplex reverse transcription–polymerase chain reaction (RT-PCR), 2 groups of diarrheal viruses were identified. The first group included astrovirus, norovirus, and sapovirus; the second group included rotavirus and adenovirus (*5*). Sapovirus polymerase region was also amplified to identify recombinant sapovirus by using primers P290 and P289 (*6*). To eliminate the possibility of co-infection of 2 different sapovirus genotypes, to localize the potential recombination site, and to understand a possible recombination mechanism of recombinant sapovirus, flanking polymerase and capsid regions, with their junction of HU/5862/Osaka/JP, were amplified with primers P290 and SLV5749 to produce a 1,162-bp product (*5*,*6*). Products were directly sequenced, and capsid- and polymerase-based phylogenetic trees showed recombinant sapovirus.

The fecal specimen was positive for sapovirus. HU/5862/Osaka/JP clustered into the genogroup I genotype 8 (GI/8 the 8/DCC/Tokyo/JP/44 cluster) (Figure) by using the recent sapovirus capsid region classification (*7*). HU/5862/Osaka/JP with GI/8 capsid was classified into GI/1 (the Sapporo/82 cluster) when polymerase-based grouping was performed. When the sequence of HU/5862/Osaka/JP was compared with that of Sapporo/82 by using SimPlot Version 1.3 (available from http://sray.med.som.jhmi.edu/SCRoftware/simplot), the recombination site was identified at the polymerase-capsid junction. Before this junction, sequences of HU/5862/Osaka/JP and Sapporo/82 were highly homologous. However, homology between them was notably different after the junction, with a sudden drop in the identity for HU/5862/Osaka/JP. By using ClustalX, HU/5862/Osaka/JP shared a 96% identity in polymerase sequence and an 85% identity in capsid sequence with Sapporo/82. In contrast, homology was 99% in the capsid region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44. Since a polymerase sequence of 8/DCC/Tokyo/JP/44 was not available in GenBank because of the unsuccessful amplification, homology in the polymerase region between HU/5862/Osaka/JP and 8/DCC/Tokyo/JP/44 was unknown.

Altogether, the findings underscored that HU/5862/Osaka/JP represented a novel, naturally occurring, recombinant sapovirus with GI/8 capsid and GI/1 polymerase. To determine whether the child was infected with this novel recombinant sapovirus or whether the novel recombinant sapovirus resulted from co-infection with 2 different viruses, Svppo (Sapporo/82-specific primer), Svdcc (8/DCC/Tokyo/JP/44-specific primer), and SLV5749 were used to amplify the capsid region (*5*). However, no amplicon was found. These negative results indicate no co-infection in this child.

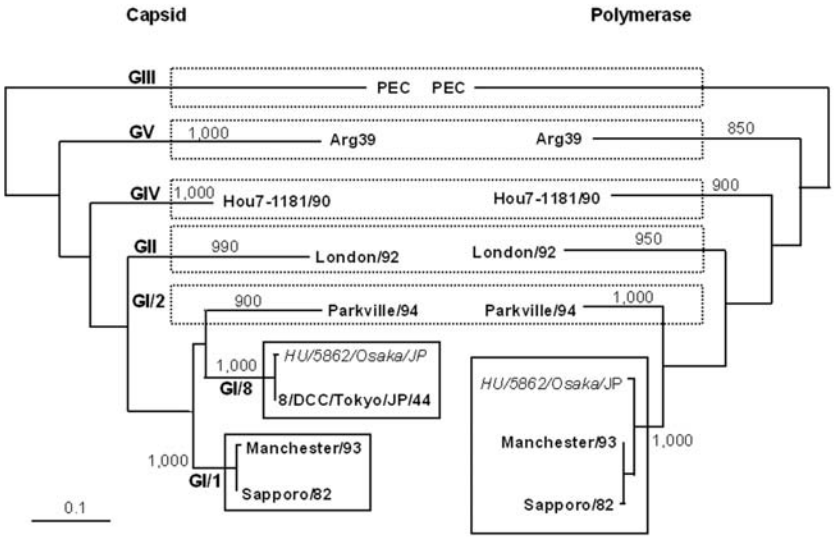

Figure. Changing genotypes of sapovirus on the basis of phylogenetic trees. Trees were constructed from partial amino acid sequences of capsid and polymerase of HU/5862/Osaka/JP highlighted in *italics*. Phylogenetic tree with 1,000 bootstrap resamples of the nucleotide alignment datasets was generated by using the neighbor-joining method with ClustalX. The genetic distance was calculated by using Kimura 2-parameter method (PHYLIP). The scale indicates amino acid substitutions per position. The numbers in branches indicate bootstrap values. Porcine enteric calicivirus was used as an outgroup strain for phylogenetic analysis. The nucleotide sequence data of sapovirus strain HU/5862/Osaka/JP has been submitted to GenBank and has been assigned accession no. DQ318530. Reference sapovirus strains and accession nos. used in this study were as follows: PEC (AF182760), London/92 (U95645), Arg39 (AY289803), Parkville/94 (U73124), Manchester/93 (X86560), Sapporo/82 (U65427), Hou7-1181/90 (AF435814), and 8/DCC/Tokyo/Japan/44 (AB236377).

Even though many molecular epidemiologic studies on sapovirus infection have been performed worldwide, reports documenting recombination in sapovirus are still limited. The first recombinant sapovirus identified was the Thai isolate Mc10 or the Japanese isolate C12 (*8*); the Japanese isolate Ehime1107 and the SW278 isolate from Sweden were identified later (*9*). Recombination occurred only in sapovirus genogroup II, which is more capable of recombination than other genogroups (*8*,*9*). In this study, we identified HU/5862/Osaka/JP with a novel recombination between 2 distinct genotypes within genogroup I. This is the first report of acute gastroenteritis caused by recombinant sapovirus genogroup I. The findings underscore that natural recombination occurs not only in sapovirus genogroup II but also in genogroup I.

In recent studies of sapovirus recombination, evidence for the location of the recombination event is lacking because of the distant geographic relationship of parent and progeny strains. HU/5862/Osaka/JP shared the closest sequences of polymerase and capsid with Sapporo/82 and 8/DCC/Tokyo/JP/44, respectively. Sapporo/82 was first isolated in 1982, and 8/DCC/Tokyo/JP/44 was isolated in 2000, both in Japan. Possibly, Sapporo/82 and 8/DCC/Tokyo/JP/44 were parental strains of HU/5862/Osaka/JP, and the event leading to the novel recombination might have occurred in Japan.

The capsid region was used for genotype classification of sapovirus (*7*). When capsid-based grouping was performed, HU/5862/Osaka/JP distinctly belonged to genotype 8. When polymerase-based grouping was

performed, HU/5862/Osaka/JP distinctly belonged to genotype 1. Therefore, sapovirus classification based on capsid sequence is questionable. We suggest that sapovirus classification should rely not only on capsid sequence but also on polymerase sequence.

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Postmortem Confirmation of Human Rabies Source

To the Editor: Rabies is a fatal encephalitis caused by a neurotropic RNA virus of the family *Rhabdoviridae*, genus *Lyssavirus*. The predominant rabies virus reservoir hosts are bats and carnivores. Among these, rabid dogs represent a substantial public health problem, particularly in developing countries (*1*).

Laboratory diagnosis of rabies is essential to guide control programs, epidemiologic surveys, and prophylactic measures (*2*). Among the laboratory tests recommended by the World Health Organization (WHO), the fluorescent antibody test (FAT) is the accepted standard for rabies diagnosis (*1*). Although rabies virus antigens can be detected in decomposed samples, FAT is less effective when such samples are tested. In those cases, polymerase chain reaction (PCR) can provide better results (*3*). Since the degree of decomposition at which FAT starts to become ineffective is unknown (*4*), when smears from decomposed samples are made for FAT, a suspension of the same brain tissues should be made in the appropriate diluents for the mouse inoculation test (MIT), cell culture, or reverse transcription–polymerase chain reaction (RT-PCR) (*2*). However, if all test results are negative, rabies cannot be ruled out because of the condition of the sample.

On February 28, in the city of Carbonita, Minas Gerais State, in southeastern Brazil, a 62-year-old man was bitten by a bat on the right ankle. Approximately 50 days later, his leg began to feel numb, and he experienced a continuous headache, pain at the site of the bite, convulsions, frequent urge to clear his throat, hiccups, nausea, difficulty in swallowing, dry lips, slightly elevated body temperature (37°C–37.5°C), paralysis of superior and inferior left limbs, shaking, and hallucinations. On May 4, 16 days after clinical manifestations began, the patient died; the cause of death was registered as a cerebral vascular accident. One month later, the body was exhumed to obtain a sample from the central nervous system (CNS), which was sent to Instituto Pasteur, São Paulo, registered as sample 5341 M/04 and tested by FAT, MIT, and RT-PCR.

In total, 8 smears were prepared from the sample to be analyzed by FAT according to the method of Dean et al. (*5*) with fluorescein isothiocyanate–labeled polyclonal antinucleocapsid antibodies. MIT was carried out as described by Koprowski (*6*) with 7 mice. For RT-PCR, RNA was extracted from the CNS sample with TRIzol, according to the manufacturer's instructions (Invitrogen, Rockville, MD, USA). RT-PCR was carried out with modifications as described by Orciari et al. (*7*), with primers 504 (sense) and 304 (antisense), aiming at the amplification of a 249-bp fragment of rabies virus nucleoprotein (N) gene, by using Superscript II (Invitrogen) and Taq DNA-polymerase (Invitrogen).

Fluorescent inclusions were observed in 6 of the 8 slides prepared for the FAT. The RT-PCR of the RNA

sample resulted in amplicons of the correct size (249 bp), as did the positive control sample, CVS strain rabies virus. No bands were observed in the reaction corresponding to the negative/reagent control (ultra-pure water). The MIT results were negative. Because the virus could not be isolated, antigenic typing with monoclonal antibodies could not be performed.

The fragment obtained in the RT-PCR was bidirectionally sequenced with DYEnamic ET Dye Terminator (Amersham Biosciences, Piscataway, NJ, USA) in a MegaBACE DNA sequencer (Amersham Biosciences) and resulted in a 165-nucleotide sequence. The final sequence was aligned with homologous sequences from GenBank by using the ClustalW (available from http://ww.ebi.ac.uk/clustalw) and Bioedit software (Isis Pharmaceuticals, Carlsbad, CA, USA). The phylogenetic tree was produced by using the neighbor-joining DNA-distance method and the Kimura 2-parameter model with 1,000 bootstrap replicates in Mega 2.1 (version 2.1) (available from http://www.megasoftware.net/). The sequence was segregated in the variant 3 cluster (*Desmodus rotundus*–related variants), which suggests that *D. rotundus* is the most probable source of infection (Figure). The sequence was assigned GenBank accession no. DQ177278.

The lack of diagnosis or delay in diagnosis can increase the number of persons potentially exposed to rabies virus infection by contact with the patient or even by organ transplantations (*8*). Moreover, an early diagnosis can decrease the cost of treatment by eliminating the use of ineffective drugs and unnecessary diagnostic tests (*2*), as well as allowing potentially useful emerging therapeutic strategies to be used (*9*).

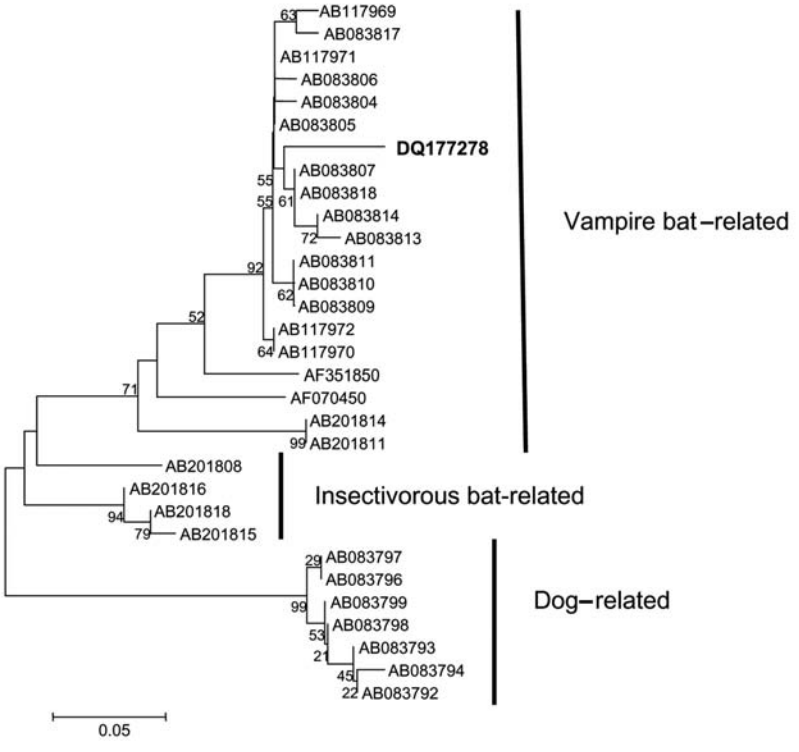

Figure. Neighbor-joining phylogenetic tree to a stretch of the 3′ end of the N gene of rabies virus variants related to vampire bats, insectivorous bats, and dogs. Strain DQ177278 is shown in **bold**. The bar indicates the genetic distance scale. Numbers at each node indicate 1,000 replicates of bootstrap values.

Before this report, no reference of a rabies diagnosis by FAT or RT-PCR had been reported from a human exhumed 30 days postmortem. The RT-PCR results agree with those obtained by David et al. (*10*) from a decomposed sample of animal origin after 36 days.

These facts demonstrate that rabies should be considered in cases of encephalitis with the classic clinical signs and symptoms as well as the paralytic form of disease (paresis and paralysis). Rabies should be suspected when early clinical symptoms, for example, itching and paresthesia, are demonstrated at the local site of infection. In addition, the laboratory investigation showed that molecular methods such as RT-PCR and sequencing were sensitive assays for nucleic acid detection and determination of the rabies virus variant in this unusual case from an exhumed human.

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Potential for Zoonotic Transmission of *Brachyspira pilosicoli*

To the Editor: Anaerobic intestinal spirochetes of the genus *Brachyspira* colonize the large intestine (*1*). Most *Brachyspira* species have a restricted host range, whereas *Brachyspira* (formerly *Serpulina*) *pilosicoli* colonizes a variety of animal and bird species and humans. *B. pilosicoli* is an important colonic pathogen of pigs and chickens (*2*). It occurs at high prevalence rates in humans in developing countries and in male homosexuals and HIV-positive persons in industrialized countries (*3*). Its potential as a human pathogen was emphasized after its identification in the bloodstream of a series of debilitated persons (*4*).

B. pilosicoli isolates from humans and other species have been used experimentally to colonize chicks, piglets, and mice (*5–7*). While these results indicate that the *B. pilosicoli* strains used lacked host-species specificity, few data exist on whether natural zoonotic spread of *B. pilosicoli* strains occurs. In 1 study that used pulsed-field gel electrophoresis (PFGE) to type isolates from Papua New Guinea, 2 dogs were colonized with *B. pilosicoli* isolates with the same PFGE types as those from villagers. However, the higher prevalence of colonization with *B. pilosicoli* in humans than dogs suggested that the dogs were infected with human isolates, probably through consumption of human feces (*8*).

Multilocus enzyme electrophoresis (MLEE) has been used to study variation in *B. pilosicoli* isolates; most studies have focused on isolates from only 1 or 2 host species (*8–10*). Generally, *B. pilosicoli* isolates are diverse, and a lack of linkage disequilibrium in the MLEE data for human isolates suggests that the species is recombinant (*8*).

We used MLEE to investigate relationships between 107 *B. pilosicoli* isolates of diverse geographic and host-species origins and the *B. aalborgi* type strain (NCTC 11492^T). Isolates were selected on the basis of their diverse origins and availability in the Murdoch University culture collection. They originated from feces of 34 pigs, 19 chickens, 13 ducks, 1 rhea, 25 humans, and 4 dogs; from 7 human blood samples; and from 4 water sources frequented by waterfowl. Isolates originated from Australia, Canada, France, Italy, the Netherlands, Oman, Papua New Guinea, the United Kingdom, and the United States.

The MLEE method used was as previously described (*8–10*); the electrophoretic mobility of 15 constitutive enzymes was analyzed. Variations in electrophoretic mobility were interpreted as representing products of different alleles at each enzyme locus. Isolates with identical enzymatic profiles at 15 loci were grouped into an electrophoretic type (ET). Genetic distance between ETs was calculated as the proportions of loci at which dissimilar alleles occurred. PHYLIP version 3.51c (Phylogeny Inference Package, University of Washington, Seattle, WA, USA) was used to analyze data and generate a dendrogram by using the unweighted pair-group method with arithmetic mean clustering fusion strategy. Genetic diversity (h) was calculated for the number of ETs as $(1 - \Sigma pi2)(n/n - 1)$, where pi is the frequency of the indicated allele and n is the number of ETs.

B. pilosicoli isolates were divided into 80 ETs (mean 1.35 isolates per ET) (Figure). *B. aalborgi* NTCC 11492^T was distinct in ET81. The *B. pilosicoli* isolates were diverse, with an h value of 0.41. Generally, they did not cluster according to host species of origin, and isolates from a given species were distributed throughout the dendrogram. Isolates from birds were more diverse than those from humans and pigs. Eight ETs contained multiple isolates, in each case from the same host species (either chickens or pigs). In 4 cases these originated from different countries: ET47 contained 2 Australian porcine isolates and 2 from the United States; ET53 contained 2 Australian porcine isolates and Scottish porcine type strain P43/6/78^T; ET54 contained 2 Australian and 2 Canadian porcine isolates; ET65 contained 1 Dutch and 1 US chicken isolate.

Although human isolates did not share an ET with isolates from other species, they were frequently closely related, differing in 1 allele. This occurred with US and Australian pig

Figure. Dendrogram showing relationships between 107 isolates of *Brachyspira pilosicoli* originating from various host species located in electrophoretic types (ETs) 1–80 and *B. aalborgi* NCTC 11492T located in ET81.

isolates in ET47 and a human isolate from Oman in ET48; an Australian pig isolate in ET61 and a UK human isolate in ET62; an isolate from an Australian HIV-positive person in ET64, and 1 Dutch and 1 US chicken isolate in ET65; and a Papua New Guinea canine isolate in ET68 and a French human blood isolate in ET69.

The distribution continuum of isolates of diverse host species and geographic origin was consistent with a lack of species specificity and suggests that *B. pilosicoli* isolates naturally have the potential to be transmitted between species. Even should there be some unexpected species-specific barrier preventing "true" animal or bird isolates from colonizing humans, animals have been colonized by human isolates, and thus could act as a reservoir of these for subsequent retransmission to humans. The results suggest that zoonotic transfer of *B. pilosicoli* isolates likely occurs in nature, e.g., after exposure to infected animals or birds, their feces, or contaminated water.

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Drug-resistant *Mycobacterium tuberculosis*, Taiwan

To the Editor: Global surveillance of drug resistance has shown that a substantial proportion of tuberculosis (TB) patients are infected with drug-resistant *Mycobacterium tuberculosis* strains (*1*). Earlier hospital-based surveys have been undertaken in Taiwan, but these lacked systematic sampling and testing methods, which made interpreting results difficult. The combined treatment efficiency and the actual prevalence of drug resistance were unknown. Thus the Taiwan Center for Disease Control initiated the Taiwan Surveillance of Drug Resistance in Tuberculosis program in 2002.

A laboratory surveillance system was established and supervised by the national reference laboratory. The system includes 6 medical centers, 2 TB referral centers, and 1 regional hospital, distributed in 4 regions of Taiwan. The 9 laboratories provide services for healthcare facilities in their own and surrounding areas. Both the national reference laboratory and contract laboratories participated in an external quality proficiency test provided by the College of American Pathologists and the national reference laboratory. Performance was also assessed by the supranational reference laboratory in Antwerp, Belgium.

The population in the first year (2003) of the survey was 22,562,663, the number of confirmed TB cases was 15,042, the estimated incidence was 66.7 per 100,000 population, and the rate of notification of new positive sputum samples was 34.6% (*2*). A total of 3,699 isolates, ≈50% of *M. tuberculosis* strains isolated, underwent antimicrobial drug susceptibility testing in the system. Since clinical data were not available, only combined (primary plus acquired) drug resistance rates were analyzed. The survey showed that the combined drug resistance rates were 9.5% to isoniazid, 5.8% to ethambutol, 6.4% to rifampin, 9.6% to streptomycin, 20.0% to any drug, and 4.0% to multiple drugs. Resistance to any single drug was 12.3%, to any 2 drugs was 4.8%, to any 3 drugs was 2.2%, and to any 4 drugs was 0.7%. In the third global drug resistance surveillance report, the median prevalence of combined drug resistance was 6.6% to isoniazid, 1.3% to ethambutol, 2.2% to rifampin, 6.1% to streptomycin, 10.4% to any drug, and 1.7% to multiple drugs (*1*).

Available historical data from Taiwan are not directly comparable because of different sampling methods and because susceptibility testing methods have been applied in various hospital settings over time (Table, available online at http://www.cdc.gov/ncidod/EID/vol12no05/05-1688.htm#table), which limits our ability to monitor trends. The latest drug resistance rates obtained from Chest Hospital, a specialized TB referral hospital, showed that the combined drug resistance of any and multiple drugs were 27.6% and 15.8%, respectively, from January 2002 to June 2004 (unpub. data).

In Taiwan, isoniazid and rifampin were introduced in 1957 and 1978, respectively. Rifampin resistance was first seen in Taiwan in 1982. In recent decades, however, the rates of primary rifampin resistance have increased (online Table), and primary resistance to multiple drugs has increased to 2.4% over time.

Based on patient data collected from Chest Hospital, multidrug resistance occurred in 42.2% of retreated TB patients, and 1.8% of multidrug-resistant isolates were found in new TB patients from January 2002 to June 2004 (unpub. data). In the third global drug resistance surveillance report, the median prevalence of multidrug resistance was 7.0% (highest 58.3%) among retreated cases and 1.1% (highest 14.2%) among new cases.

Significant declining trends were observed for any acquired resistance (67.0% to 42.6%, $p<0.0001$) and acquired multidrug resistance (46.0% to 24.6%, $p<0.0001$) at the Taiwan Provincial Chronic Disease Control Bureau from 1996 to 2001 (*3*,*4*). In addition, a decline in combined isoniazid resistance (43.1% to 16.4%, $p<0.0001$), rifampin resistance (23.4% to 9.5%, $p<0.0049$), and multidrug resistance (18.2% to 7.8%, $p<0.0113$) was also reported from Kaohsiung Medical University Hospital from 1996 to 2000 (*5*). Taken together, data obtained from the Taiwan Surveillance of Drug Resistance in Tuberculosis and those reported previously show that rates of combined resistance to any drugs and multiple drugs has declined in Taiwan.

For retreated cases, the high acquired resistance rates indicated suboptimal initial treatment and insufficient case management of new patients, which raises a challenge to the National TB Control Programme in Taiwan. The direct observed treatment, short-course (DOTS) strategy has consequently been suggested to expand to all patients with newly diagnosed cases. The Taiwan Surveillance of Drug Resistance in Tuberculosis program will be extended to collect each patient's clinical and epidemiologic data, according to principles suggested in the guidelines prepared by the World Health Organization.

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Enrofloxacin in Poultry and Human Health

To the Editor: Following logic similar to that recently used by the US Food and Drug Administration to withdraw approval for enrofloxacin, a recent letter estimated that fluoroquinolone use in poultry could compromise responses to antimicrobial drugs in >24,000 persons per year in the United States (*1*). However, >99.9% of this estimated risk appears to result from incorrect assumptions. Potentially important corrections include the following: 1) not attributing resistance from foreign travel and human ciprofloxacin use to domestic use of enrofloxacin in poultry (this could reduce the estimated risk by ≈1/3) (*2*); 2) updating the estimated fraction of human foodborne *Campylobacter* infections caused by poultry to reflect declines in microbial loads on chicken carcasses since 1992 reduces the estimated risk by a factor of perhaps 1/10 (*3*) (the cited 90% estimate by Hurd et al. [*1*] was intended for use as part of a conservative upper-bounding analysis, not as a realistic point estimate); 3) replacing an assumption that 10% of infected persons would benefit from antimicrobial drug therapy with a more data-based value of 0.6% (*4*) would reduces the estimated risk by a factor of 0.6/10 = 0.06; 4) replacing an assumption that fluoroquinolones are prescribed for *all* affected patients receiving antimicrobial drug treatment (rather than, for example, erythromycin) by a more realistic value of fluoroquinolones being prescribed for perhaps ≈50% of patients (*2*) reduces the estimated risk by a factor of ≈50%; 5) replacing an assumption that *all* such cases lead to compromised responses with a more data-driven estimate that perhaps ≈17% of patients have compromised responses would reduce the estimated risk by a factor of 1/6 (*5*); and 6) recognizing that reducing enrofloxacin use may not decrease fluoroquinolone resistance in all *Campylobacter* spp. from food animals (effect not quantified) (*6*). Together, such changes reduce the estimated risk by a factor of at least (1/3) × (1/10) × (0.6/10) × (1/2) × (1/6) = 0.00017, or by >99.9%.

More notably, the calculation in (*1*) also wrongly assumes that the fraction of patients with fluoroquinolone-resistant infections times the fraction of infections caused by poultry gives the fraction of patients with compromised response caused by fluoroquinolone use in poultry. As a simple counterexample, suppose that 80% of all infections were caused by poultry, with the rest caused by something else (e.g., water), and that all and only the 20% of infections caused by the latter source are resistant. Then the procedure in (*1*) would estimate (80% of infections caused by poultry) × (20% of infections resistant) = 16% as the fraction of resistant infections caused by poultry, even though the correct answer is zero. Thus, the basic logic of the calculation is flawed.

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In response: Cox's letter (*1*) contains a number of false assumptions, errors, misleading assertions, and misquotations. Cox asserts that annually 1 person or fewer in the United States will experience an adverse effect because of fluoroquinolone use in poultry. He reduces 10-fold my referenced risk for persons acquiring *Campylobacter* infections from poultry (*2*). His unrealistically low estimate is not given in his referenced citation. His estimated risk is also much lower than in the reference 2, which Cox himself quotes, "Poultry is the most common cause of sporadic cases of campylobacteriosis in the United States" (Economic Research Service of the US Department of Agriculture) (*3*). Cox knows that his assertion (*4*) that poultry make little or no contribution to human *Campylobacter* infections has been extensively examined and found to be wrong. Indeed, an entire section in a recent US Food and Drug Administration (FDA) determination was written about the unreliability of Cox's testimony and these assertions, a finding made by both the FDA commissioner and an administrative law judge (*5,6*).

Cox also misquotes Busby et al. (*3*) when he asserts that only 0.6% of persons with *Campylobacter* infections benefit from antimicrobial drugs. The Busby article states that 0.6% of persons with *Campylobacter* infections need "hospitalization," not how many would benefit from antimicrobial drug therapy. Cox has thus made a misleading attribution (something he has previously been found to do [*5*]).

Busby et al. (*3*) estimated that in 1993, ≈1,500,000 persons in the United States acquired *Campylobacter* infections from food sources. Even if the proportion who can benefit from receiving antimicrobial drugs is as low as 2%, this translates to 30,000 persons. If 20% of these infections were caused by fluoroquinolone-resistant *Campylobacter* spp., then 6,000 persons would potentially have their therapy and outcome compromised, rather than the 1 person that Cox would have us believe. More realistic is the figure of 24,000 persons estimated previously to be at risk of having an adverse outcome (or ≈285 persons for every 1 million chickens treated with fluoroquinolones) (*1*). Cox's assumptions and calculations thus seem flawed and unrealistic.

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Biodefense Shield and Avian Influenza

To the Editor: In defending against avian influenza virus H5N1, the possibility of adopting treatments being developed for biodefense should not be overlooked. Biodefense medicine primarily concerns respiratory infections because bioweapons in their deadliest form disperse *Bacillus anthracis* and *Yersinia pestis*, the causes of anthrax and plague, and highly contagious viruses like smallpox, Ebola, and Marburg as aerosols. The National Institutes of Health and Department of Defense have funded developing novel biodefense medications designed to stimulate innate mucosal immunity by using interferons (IFNs) and interferon inducers.

We suggest that studies begin immediately to explore the potential of IFNs to prevent infections and reduce deaths caused by avian influenza viruses in animal models and humans.

Modulating innate mucosal immunity is promising as a rapid-acting, broad-spectrum approach to combat bioterrorism (*1*). Innate immunity, the initial response to a pathogen, is potentially capable of eradicating infection. Even when the innate immune response cannot eliminate a virus, it may substantially reduce viral load, reduce pathology, facilitate clearing of the virus by the adaptive immune response, and slow the spread of infection (*1*). As biodefense medications, IFNs and IFN-inducers are under development for aerosolized delivery to the lungs (*2*,*3*). Conventional IFN administration by injection often results in low concentrations at target sites and high concentrations in circulation, which may cause serious side effects. Aerosolized delivery minimizes side effects and produces more rapid clinical responses. Inhaled IFNs have proven to be well tolerated and beneficial for rhinovirus infection (*4*) and pulmonary tuberculosis (*5*).

Medications being developed to prevent infections caused by viral bioweapons and other diseases include 1) Oral IFN-α or Alferon low dose oral (LDO) (Hemispherx Biopharma, Inc., Philadelphia, PA, USA); 2) inhalable IFN-γ (InterMune, Brisbane, CA, USA); 3) dsRNA [Poly (ICLC)] or Ampligen (Hemispherx Biopharma, Inc.); 4) ssRNA (Aldara and Resiquimod from 3M Pharmaceuticals, St. Paul, MN, USA); and 5) CpG7909 and CpG10101 oligonucleotides (Coley Pharmaceutical Group, Wellesley, MA, USA) (*2*). These drugs have either been approved by the Food and Drug Administration (FDA) (Aldara), are in clinical trials (Alferon LDO, inhalable IFN-γ, Resiquimod, CPG7909, and CpG10101), or at a preclinical stage of development (Ampligen). Aldara is approved for genital warts, actinic keratoses, and basal cell carcinoma. Others drugs are being tested for aerosolized delivery to modulate mucosal immunity of the respiratory tract. All could be expeditiously tested with inhalational or intranasal administration in H5N1 models with mice, ferrets, pigs, and monkeys.

IFN-α and IFN-γ work by binding their receptors and activating downstream antiviral pathways involving the dsRNA-dependent protein kinase (PKR), the 2′, 5′ oligoadenylate synthetase/RNase L, or the MxA protein. dsRNA, ssRNA, and CpG oligonucleotides are ligands for toll-like receptors (TLRs) and modulate antiviral immunity through TLR signaling pathways and IFN induction (*2*). At the cellular level inside the lungs, these drugs will enhance phagocytotic and cytolytic activity in alveolar macrophages.

Once infection is established, H5N1 resists the antiviral effects of IFNs and tumor necrosis factor-α (*6*). Resistance is associated with the nonstructural gene of H5N1 and may be 1 mechanism for H5N1's extraordinary virulence. Therefore, prophylactic use of IFNs and IFN-inducers is critical to combat H5N1. They may also be effective if administered immediately after infection.

IFN resistance also exists for other viral infections. For instance, poxviruses including vaccinia virus encode 2 proteins that interfere with RNaseL and PKR pathways and 2 soluble IFN receptors that interfere with IFN-induced antiviral pathways. Nevertheless, at least in animal models, pre-infection administration of exogenous IFN can reduce deaths and poxvirus viral load. In mice, intranasal administration of IFN-α and IFN-γ prevents lethal vaccinia infection (*3*). IFN-α, IFN-γ, and an IFN inducer, Poly (ICLC), protect mice infected with H1N1 influenza virus (*7*). Hence, we suggest that anti-H5N1 prophylaxis by IFN-stimulated innate mucosal immunity is a promising therapy worth immediate investigation in animal models.

A second mechanism proposed to explain H5N1 virulence is also IFN related. This is the "cytokine storm," as shown by elevated levels of proinflammatory cytokines including IFNs found in 2 patients who died of H5N1 infections (*8*). Cytokine storms can result in autoimmune reactions, tissue damage, or septic shock. High IFN doses for long periods may exacerbate autoimmunity. However, despite similar cytokine storms (*9*), some severe acute respiratory syndrome patients respond well to IFN therapy (*10*). Optimal formulation and regimen of IFN administration could be crucial to effective anti-H5N1 prophylaxis. In the interests of safety, we propose that initial prophylaxis studies use relatively low IFN doses for short periods (≈1–2 weeks).

It is unlikely that all of these drugs will effectively protect against H5N1. And a drug that is effective might not work for everyone; genetic polymorphism influences IFN response. However, FDA approval of even one of them might save many lives.

Acknowledgments

We thank Tom Hollon for his editing and helpful suggestions.

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Our company, AFG Biosolutions, Inc., has no grants, contracts, or other financial support to develop a commercial antiinfluenza product from among the immunomodulators mentioned here.

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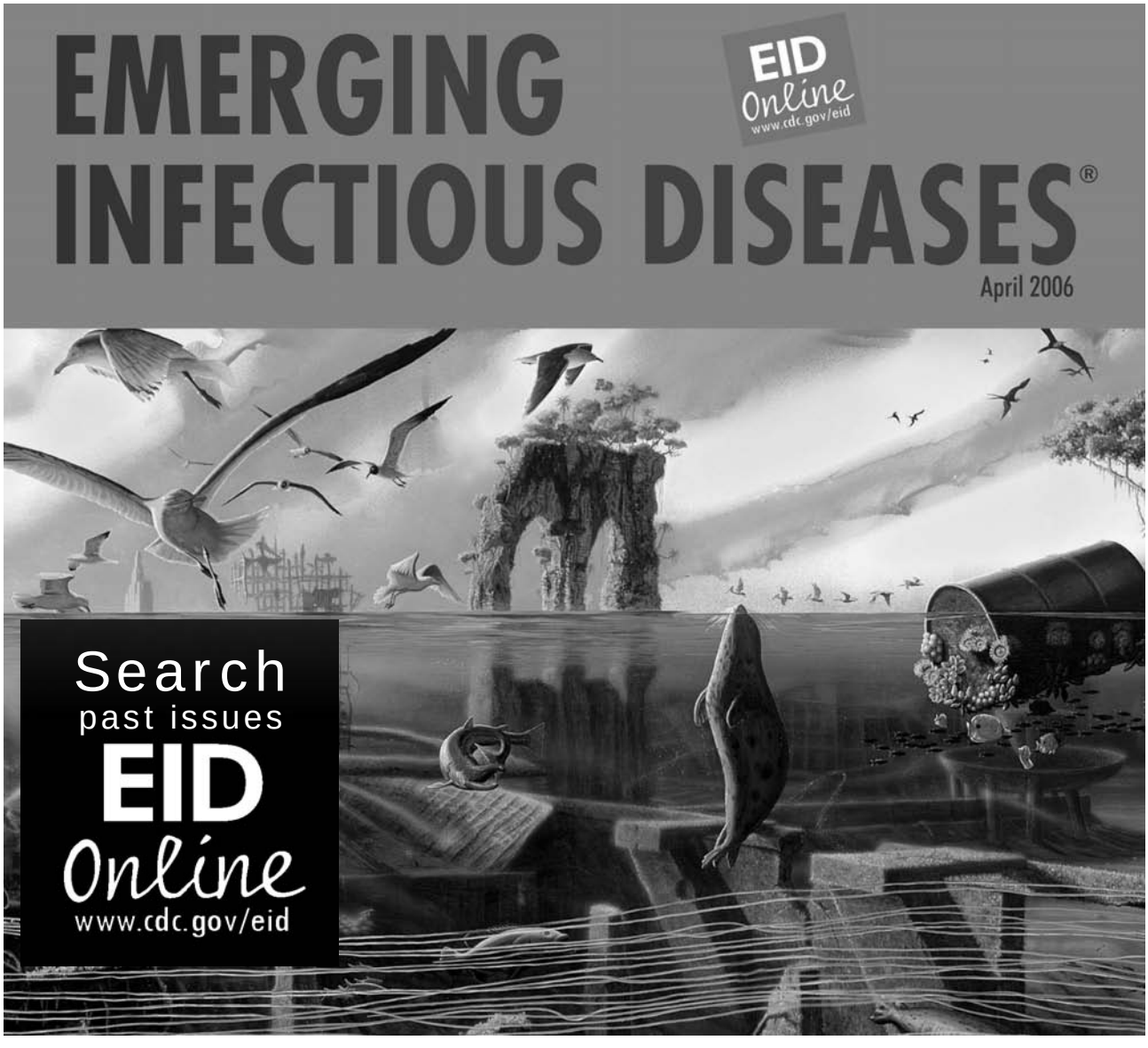

René Dubos, Friend of the Good Earth: Microbiologist, Medical Scientist, Environmentalist

Carol L. Moberg
ASM Press, Washington DC, 2005

ISBN: 1555813402
Pages: 260; Price: US $29.95

René Dubos: Friend of the Good Earth is the only biography that documents Dubos's early life and progression into one of the 20th century's great scientist-philosophers. It is well researched and adequately referenced, when references are available. This book will be an invaluable resource, with some caveats.

This book suggests that philosophy came first and science followed. In reality, The White Plague, Mirage of Health, Torch of Life, Man Adapting, and So Human an Animal were written after the seminal scientific work had been done or while it was in progress. In science, Dubos never made exaggerated claims or overstepped scientific evidence. In his books and lectures, however, he could expand his thoughts, ideas, and hunches without recrimination.

The years of Dubos's scientific career after his early success culturing *Mycobacterium tuberculosis* in dispersed culture and the less-than-successful attempts to develop diagnostic tests are given little attention. Many years of work on acid-fast organisms did little to expand knowledge of tubercle bacilli, but the work opened avenues of research on the host's responses to infection. The use of living and killed bacillus Calmette-Guérin and endotoxin aided our understanding of the multiplicity of immune responses.

Moberg states that Walsh McDermott and the Cornell group grew 50 L of tubercle bacilli every day; while harvesting the organisms, one of the investigators became ill. This statement is wrong in 2 respects. First, to grow 50 L per day would take a pilot plant, and second, the work was done in Dubos's laboratory. Every week in Dubos's laboratory, 50 L media was inoculated so a mass of tubercle bacilli surface growth could be obtained. Four weeks later, the 50 L surface culture was harvested by using a number 2 centrifuge fitted with a basket filter. Opening the centrifuge aerosolized killed bacilli. After several exposures, a colleague had a severe generalized sensitivity reaction. Then the tubercle bacilli hit the fan! The old centrifuge was put into a specially constructed wooden box (limed oak, to match the rest of the laboratory cabinets), with a large, gasketed door and rubber gauntlet gloves to allow manipulation of the centrifuge, and the entire cabinet was vented to the roof. No one became ill again.

In another minor error, a facility known as Mousehatten House was designed to easily feed and care for large numbers of animals on special diets, so that their gut flora could be evaluated. Each animal would have its own unit with food, drinking fountain, and waste collection. Plans for the construction had to be submitted to New York City licensing and inspection authorities because it was a new animal unit in a new laboratory. Unfortunately, the plans were returned as inadequate—no provision for fire escapes. Of course, Dubos thought this story was hilarious, and all of us recounted it at various gatherings. After the story appeared in the New Yorker, I found the front portion of an old Christmas card depicting a toy train on my desk. On the back Dubos had written, "'When we are no longer children we are already dead'—Brancusi" and "'Genius is childhood recaptured'—Baudelaire."

The creation of a specific-pathogen-free mouse by Nelson and Collins gave the Dubos group a new and reliable standardized "fuzzy test tube." These animals provided the impetus for work on gut flora, the association of microbes with mucosal surfaces, and some of the earliest work with probiotics. These animals were also used in Dubos's students' social science and crowding experiments. It was a time of scientific advances and the foundation for much of Dubos's work in philosophy. This decade of Dubos's scientific work is glossed over in a cursory manor. The author even quotes one of Dubos's colleagues as stating that the work isn't worth a "hill of beans."

Also, some of Dubos's great talents are not mentioned. He did not like statistics and avoided using them by repeating his experiments over and over, deleting parameters that seemed fruitless and adding others that seemed promising. This repetition gave his experiments numbers that were large enough not to need statistical analysis, and none of his published articles ever had to be retracted. His conclusions were based on the data at hand; he never overstepped this boundary. Dubos also had the ability to analyze raw data, focusing on important aspects and suggesting new and fruitful experiments.

This book often mentions Dubos's farm in Garrison, New York. Planting trees and hunting for water were among his prime pleasures. Behind his house was a beautiful grotto in which grew a lovely hepatica. His neighbor, an elderly European farmer, told him, "There's a spring here. Drill through that rock and you will find water." Dubos began to drill with diligent, laborious work, and after ≈6 months, he had drilled a 1.5-inch hole, 30 inches deep into the rock without finding water. During all these hours of labor, Dubos was always thinking about experiments, contemplating what he would do that week in the

laboratory, or honing his thoughts on a new lecture or book, so the time was not wasted. The neighbor shared Dubos's disappointment in not finding water and obtained a stick of dynamite to put into the hole. The dynamite obliterated the rock, the beautiful grotto, and the lovely hepatica, but no spring was ever found.

Dubos enjoyed wildflowers and was an expert on the identification of wildflowers of New York. He had a loose-leaf portfolio of the wildflowers of New York by the University of New York State Museum from 1921. The illustrations were lithographs of color drawings. He cut them apart and took those he had not yet seen on his walks and excursions. I dare say he saw and identified most, if not all, of the flowers in that portfolio. He was proud of this accomplishment and was delighted when the occasional guest to his farm noticed or knew of the wildflowers. (Most of his scientific colleagues were not in this league.) His passion for planting trees was great, but he did not have the strength to dig deep holes in that rocky landscape, and watering in many places was difficult. However, as in all his endeavors, he persisted, replanting new trees where previous plantings failed to survive. Most plantings were not done according to a grand plan but of necessity, following the curvature of the driveway and places where he could dig. Hemlocks and dogwoods were the species of choice.

Dubos had difficulty with some personal relationships. If he knew a particular person he did not like was in town, he would hide in his office or seclude himself in his apartment. This inability to confront a person extended to colleagues and visitors, but others were always welcome. He kept a nervous distance from people of authority. Whenever he had to report to his superiors or go to Washington to testify or present to a committee, the telltale signs appeared several days in advance. His lips became coated with a white film from constantly chewing Gelusil, and herpetic lesions appeared on his lip. These signs indicated he had to perform one of his distasteful duties.

A favorite book of Dubos's was The Unseen World, a result of the first Christmas Lectures at Rockefeller University. In this whimsical book, he gained a rapport with his audience, and they received a different understanding of life. Dubos wrote, "this microbiology as a way of life, fortunately not incompatible with more earthy ways." As one of the great scientists of the 20th century, Dubos in his later decades turned to philosophy to better spread his views on humans and their reaction to all things around them. We should remember that throughout his life, Dubos was "so human an animal."

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Andrew Wyeth (b. 1917). Christina Olson (1947)
Tempera on panel (83.8 cm × 63.5 cm). Courtesy of Curtis Galleries, Minneapolis, Minnesota, USA

On the Threshold of Illness and Emotional Isolation

Polyxeni Potter*

"I was very thin and nervous so my father and mother took me out of school and had me tutored at home," recalls Andrew Wyeth about his education after the third grade (*1*). The ailment that kept him away from his peers during childhood and adolescence was a "sinus condition," later diagnosed as tuberculosis. "I played alone and wandered a great deal over the hills, painting watercolors that literally exploded, slapdash over my pages, and drew in pencil or pen and ink in a wild and undisciplined manner" (*2*). His precocious artistic talent was also reigned in and cultivated in the home environment. He learned from his father, painter, muralist, illustrator Newell Wyeth, who studied with foremost 19th-century illustrator, Howard Pyle (*3*).

Influenced by Henry David Thoreau and the transcendentalists, Newell Wyeth promoted awareness of the metaphorical and metaphysical value in even the most mundane objects and advocated attention to and alignment with the subtleties of the natural world. He believed that "…a man can only paint that which he knows even more than intimately…. And to do that he has got to live around it, in it and be *part* of it" (*4*). His young son spent hours painting objects, only to lament that he could "never get close enough to an object or inside of it enough" (*5*), but the telescopic views of the domestic implements he painted throughout his career show this early still-life training.

At Chadds Ford, the farming village in Pennsylvania where he was born, Wyeth worked with charcoal and oils and studied the masters, among them Albrecht Dürer, an early source of inspiration. In Maine, where the family spent summer months, he experimented with watercolors and painted landscapes in the style of Winslow Homer, with whom along with Thomas Eakins, he felt strong kinship.

"They look magnificent, and with no reservations whatsoever, they represent the very best watercolors I ever saw," wrote his father about the works in 20-year-old

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Andrew's first show in New York City (*6*). The critical acclaim of this and other early shows did not satisfy the young Wyeth, who thought his work too spontaneous and facile. "I was skimming along on a very superficial level. I had a terrible urge to get deeper, closer to nature" (*7*). His brother-in-law Peter Hurd, also an artist, introduced him to egg tempera, a medium that, in combination with the dry-brush method he favored, slowed down his technique and added distinctive texture to the work.

"When he died," Wyeth said of his father, "I was just a clever watercolorist—lots of swish and swash…. I had always had this great motion toward the landscape, and so with his death, …the landscape took on a meaning—the quality of him" (*2*). The emotional content of the work intensified, and he began to paint figures, mostly portraits of single figures. He abstracted interfering elements, reducing the picture to an object or surrounding that captured their essence, while the figures themselves were no longer present. Trodden Weed (1951), a painting said to have been admired by Nikita Khrushchev, shows only a man's booted legs walking across the grass. This work, conceived while Wyeth recuperated from surgery to remove part of his lung, was intended as self-portrait.

Wyeth's work from Pennsylvania and Maine drew from an anti-modern, anti-urban sentiment prevalent in the United States between the Civil War and World War II (*8*). Era art favored idealized rural scenes as antidote to ongoing rapid social and economic change. Wyeth reinvented and reshaped this pictorial backdrop. Georgia O'Keefe, John Marin, and others in this period examined images and objects in their locales for universal metaphors. Edward Hopper and Charles Sheeler, among others, used common objects to depict the poor and dispossessed. To objects, Wyeth added intense personal associations, meaning, and emotion (*3*).

About abstract expressionism, a modern art movement (Piet Mondrian, Max Ernst) dominating the scene as Wyeth came of age, he remained ambivalent. "My aim is to escape from the medium with which I work. To leave no residue of technical mannerisms to stand between my expression and the observer…. Not to exhibit craft but rather to submerge it…" (*9*).

During one of his trips to Maine, Wyeth met Christina Olson and her brother Alvaro, who lived in a dilapidated, peaked-roof farmhouse in Cushing. Christina, disabled from poliomyelitis or some aggressive form of arthritis, had difficulty walking. Her strength and perseverance intrigued and inspired him, and during their long friendship, he kept a studio in the Olson household. Christina's World (1948), the image, from the back, of his friend in a large field crawling toward her home, became one of the most recognized American paintings. "I felt the loneliness of that figure—perhaps the same that I felt myself as a kid," Wyeth said of the work (*2*).

Christina Olson, on this month's cover, allows a more generous glimpse of the figure's profile of illness, disability, and their psychological outcome. In Wyeth's words, "a wounded gull," Christina has been left behind. Alone, she rests on the threshold, her body propped against the open door, hair blown softly in the breeze. Her posture in the center of the painting, erect and dignified, defies the somber aspect of her wasting limbs. Unable to join in, she seeks a lighted spot, an outlet into normalcy. And, "in the moment," she soaks up the sun, connecting with the universe.

Wyeth's empathetic portrait of his friend's physical impairment symbolizes the limits imposed by illness, in her case, undiagnosed and misunderstood, in his, finally named tuberculosis. Current efforts, whether skin testing of children at risk (*10*) or genotyping of *Mycobacterium tuberculosis* strains (*11*), lessen the life-defining impact of this disease and lower the threshold of illness and emotional isolation.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

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Community-acquired Pneumonia Caused by *Staphylococcus aureus*, 2003–04 Influenza Season

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Temple Monkeys and Health Implications of Commensalism, Kathmandu, Nepal

Haemophilus influenzae type b Reemergence after Combination Immunization

Unusual Brazilian Genotypes of *Toxoplasma gondii* as Cause of Human Ocular Toxoplasmosis

Co-infections of Adenovirus Species in Previously Vaccinated Patients

Human Rotavirus Serotype G9, São Paulo, Brazil, 1996–2003

VIM-1 Metallo-β-lactamase in *Acinetobacter baumannii*

Guillain-Barré Syndrome, Greater Paris Area

Francisella tularensis in Rodents from China

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Upcoming Infectious Disease Activities

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Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only 2. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., “The Study” and “Conclusions.” Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch of first author—both authors if only 2. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Book Reviews. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.