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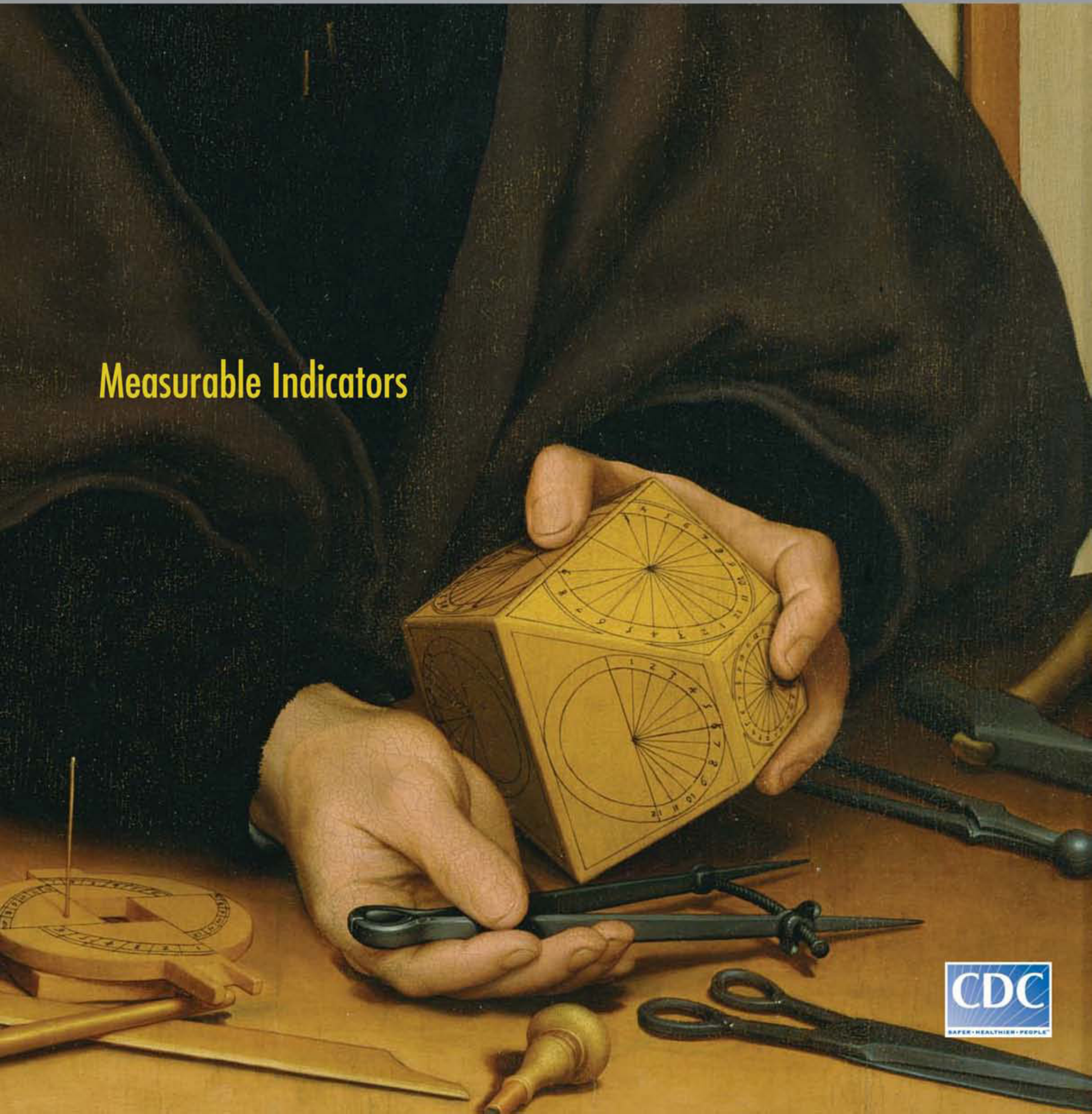

Measurable Indicators

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Hans Holbein the Younger (1497–1543).
Nicholas Kratzer (1528)

Oil on wood, 83 cm x 67 cm,
Louvre, Paris, France
Photo: Erich Lessing/
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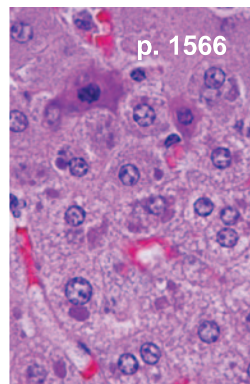

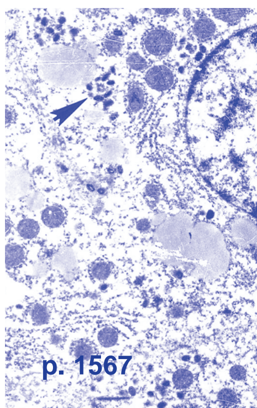

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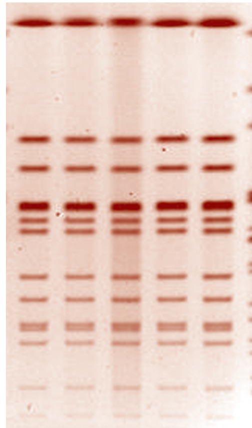

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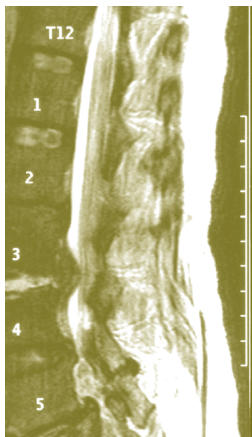

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New Measurable Indicator for Tuberculosis Case Detection

Martien W. Borgdorff*†

The World Health Organization's goal for tuberculosis (TB) control is to detect 70% of new, smear-positive TB cases and cure 85% of these cases. The case detection rate is the number of reported cases per 100,000 persons per year divided by the estimated incidence rate per 100,000 per year. TB incidence is uncertain and not measured but estimated; therefore, the case detection rate is uncertain. This article proposes a new indicator to assess case detection: the patient diagnostic rate. The patient diagnostic rate is the rate at which prevalent cases are detected by control programs and can be measured as the number of reported cases per 100,000 persons per year divided by the prevalence per 100,000. Prevalence can be measured directly through national prevalence surveys. Conducting prevalence surveys at 5- to 10-year intervals would allow countries with high rates of disease to determine their case detection performance by using the patient diagnostic rate and determine the effect of control measures.

Reversing global tuberculosis (TB) incidence by 2015 is included in the Millennium Development Goals (1). Prevalence and death rates (indicator 23) and the proportion of cases detected and cured under a directly observed treatment strategy (DOTS) (indicator 24) are used to measure progress towards this goal. For indicator 24, the World Health Organization (WHO) has formulated the following goals: a case detection rate of 70% and a cure rate of 85% (2,3). If both targets are achieved, the effect on TB transmission will be considerable (3,4).

WHO defines the cure rate as the proportion of new cases of smear-positive TB that were cured through treatment; this rate is routinely measured by treatment registers. The case detection rate is the proportion of incident smear-positive TB cases detected through a TB program. The case detection rate is measured as the notification rate of new cases of smear-positive TB divided by the estimated incidence rate.

Incidence is estimated by using various sources of information (5,6). An important element in these estimates is the proposed relationship between the incidence of TB and the annual risk for TB infection. Styblo estimated that, in the absence of control, a 1% (i.e., 1,000/100,000) annual risk for infection would correspond with an incidence of new cases of smear-positive TB of approximately 50 per 100,000 (7,8). In other words, in the absence of control measures, 50 cases would generate 1,000 infections; i.e., the average patient with a new case of smear-positive TB would generate approximately 20 infections over time. The annual risk for infection is measured imprecisely through tuberculin surveys; problems include cross-reactions caused by *Mycobacterium bovis* bacillus Calmette-Guérin vaccination and environmental mycobacteria. The relationship between risk for infection and incidence varies, depending on the quality of the control measures and the role of HIV infection (9). Deriving incidence from prevalence and the average duration of disease (6) also gives uncertain results, in particular because the duration of disease cannot be measured with precision. Deriving incidence from the number of TB deaths and estimated TB case death rates (6) also gives uncertain results because ascertaining cause of death is incomplete in most countries with a high rate of TB, and TB case death rates vary, since they depend on the quality of treatment and are strongly influenced by HIV co-infection (6). Therefore, incidence estimates are particularly uncertain in sub-Saharan Africa, which has the highest per capita TB incidence and prevalence of HIV infection in the world (5,6).

To measure the incidence of new cases of smear-positive TB directly, one would require at least two prevalence surveys, e.g., 1 year apart, as well as a surveillance mechanism to detect incident cases in patients dying or emigrating out between the first and second survey. Moreover, correct identification of persons with TB is needed to link results of the second survey to the first. If the time between surveys is reduced, this reduces the bias of patients dying or moving out, but the number of incident cases will be smaller, reducing precision. Direct measurement is thus costly and complicated, and no country is currently applying this method. As a result, the incidence of new cases of smear-positive TB is uncertain, and TB programs do not

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know whether they are reaching the case detection rate goal. This problem affects low-income countries with high rates of TB in particular, since these countries tend to have inadequate case detection and reporting systems.

These measurement problems are important because the effect of TB programs depends on their success in detecting cases. This article proposes an alternative indicator to measure TB case detection. This indicator does not directly measure the proportion of cases detected but the speed at which they are detected.

New Indicator: Patient Diagnostic Rate

Since the case detection rate is estimated indirectly and is uncertain, another indicator that can be measured more directly would be desirable. This indicator is the rate at which prevalent case-patients are recruited by TB programs, referred to here as the patient diagnostic rate. In practice, this indicator can be measured as follows: the number of newly reported cases (i.e., never treated) of smear-positive TB per 100,000 population per year (notification rate) divided by the prevalence of new cases of smear-positive TB per 100,000 population. The numerator is obtained from surveillance data and the denominator from a prevalence survey. The denominator represents the population at risk for case detection, the numerator those actually detected. At present, the proposal is to restrict patient diagnostic rate to smear-positive cases because smear microscopy is currently the most widely applied tool to confirm TB in countries with high rates of disease. The proposal is restricted to new cases, since this best captures the effects of case detection. The prevalence of previously treated TB depends strongly on the cure rate. Patient diagnostic rates in countries conducting and reporting a prevalence survey during the past decade are presented in the Table.

A more refined estimate of patient diagnostic rate may be obtained by stratification for important variables that are recorded routinely, such as age, sex, urban versus rural areas, and DOTS versus non-DOTS areas. DOTS areas are defined as those that have adopted the WHO TB control strategy. Such stratification may help identify TB priorities for strengthening case finding and assess the effect of DOTS. In countries with a high prevalence of HIV infection, separate estimates for persons with and without HIV infection indicate differences in the patient diagnostic rate and death rates between TB patients with and without HIV co-infection (6).

Patient Diagnostic Rate, Case Detection Rate, and Program Effect

The quantitative relationship between the case detection rate, patient diagnostic rate, and expected program effect depends on the way we conceive case detection. Two approaches have been used in the past, perhaps best explained with the models of Styblo (model 1) (2,3) and Dye et al. (model 2) (4).

Model 1 assumes that cases are either detected after an average of 4 months or not at all (2,3). Patients whose cases are not detected either die or self-cure after an average of 2 years. Self-cure refers to patients reverting to latent infection without being treated. In model 2 (4), cases are detected at a certain rate (patient diagnostic rate), and the patients die or self-cure at a certain rate. The proportion of cases detected in model 2 thus depends on the relative size of these two rates: the larger the patient diagnostic rate, the larger the case detection rate and the shorter the average delay. As a result of these different assumptions, the same case detection rate of 70% is associated with a larger patient diagnostic rate and a larger impact on TB prevalence in model 2 than in model 1 (Appendix). In the absence of HIV infection, a case detection rate of at least 70% corresponds with a patient diagnostic rate of at least 0.84 per person-year in model 1 and a patient diagnostic rate of at least 1.17 per person-year in model 2.

How do these model targets compare with values of patient diagnostic rates we observe in the real world? A rough, indirect estimate of patient diagnostic rate in the Netherlands is 2.5 per person-year (Appendix). Of more relevance may be the direct estimates in countries with high rates of TB (Table): the patient diagnostic rate was 0.24 in China, 0.43 in Korea, and 0.51 in the Philippines. These three countries did not meet the goal for case detection by models 1 or 2.

For the patient diagnostic rate to be a useful indicator, the best reporting rate should be obtained. For instance, if general hospitals in China, or the private sector in the Philippines and Korea, fail to notify the patients they treat, the patient diagnostic rate will be underestimated (the

Table. The patient diagnostic rate in China, Philippines, and Korea[a,b]

	Notification rate smear-+ TB per 100,000	Prevalence rate smear-+ TB per 100,000	PDR	Ref
China, 2000	17	72	0.24	10,11
Philippines, 1997	118	229	0.51	12,13
Korea, 1995	26	60	0.43	14,15

[a]TB, tuberculosis; +, postive; PDR, patient diagnostic rate; ref, reference number.
[b]In the Philippines, total prevalence was 310/100,000. Of 50 cases with drug susceptibility results and known treatment history, 37 (74%) had not been previously treated. The assumption was that 74% of prevalent smear-positive patients had not been previously treated. In Korea, total prevalence was 93/100,000. The prevalence of new smear-positive TB was obtained from the unpublished survey report.

same limitation applies to the case detection rate). Therefore, the use of patient diagnostic rate is not an alternative to a good reporting system but supports the development of such a system. If the notification system detects most cases (e.g., with a patient diagnostic rate exceeding the goal of model 2 of 1.17), then reporting data may be used exclusively to monitor trends, as is done in countries with low rates of disease.

Limitation of the Patient Diagnostic Rate

A limitation of the patient diagnostic rate is that measuring TB prevalence is complicated and costly with the current standard methods, which require the use of mobile chest radiograph equipment as a screening tool. However, this limitation can be overcome. High standard prevalence surveys have been shown to be feasible (Table). Moreover, their cost represents a small proportion of the cost of control programs. TB control programs in the 22 countries with high rates of the disease annually cost an estimated U.S. $940 million, approximately half of which is within the TB program budget, while the other half represents health infrastructure costs (16). Twenty-two national surveys, performed with current standard methods once every 5–10 years, would cost approximately U.S. $25–$50 million in total, i.e., <U.S. $10 million per year. This cost represents at most 1% of the cost of TB control programs.

Nevertheless, new survey methods, using other diagnostic algorithms or new diagnostic methods, that do not require mobile chest radiographs would be beneficial. They would promote the measurement of TB case detection and program effect in the 22 countries with high rates of disease and in other high incidence-countries with limited resources, especially Africa.

Conclusion

The patient diagnostic rate is a measurable indicator for detecting patients with previously untreated cases of smear-positive TB. The expected effect of a TB control program on transmission increases with an increasing value of this indicator. A patient diagnostic rate of >0.84 would correspond to the original WHO goal proposed by Styblo of detecting >70% of incident cases. A patient diagnostic rate of >1.17 would meet the goal of 70% case detection as used by Dye et al. to project the effect of the DOTS strategy (4). On the basis of further evidence about patient diagnostic rates and associated TB program impact, a revised goal may be formulated in the future.

While monitoring performance is extremely useful in the short-term, monitoring effects, or at least the trend of TB prevalence, is most important in the medium- and long-term. Programs aimed at reducing TB prevelance can assess whether the decrease is occurring through reporting rates, if case detection is good, or by carrying out prevalence surveys every 5–10 years, if the completeness of case detection varies or is uncertain. Prevalence surveys would provide direct information on indicator 23 for measuring progress towards meeting the Millennium Development Goals (1). Monitoring effect through prevalence surveys allows the patient diagnostic rate to be measured and the risk factors for nondetection to be identified by the health service. Developing new diagnostic methods, obviating the need for chest radiographs, would be extremely helpful for such surveys. Monitoring TB is recommended through prevalence surveys in countries with high rates of disease until reporting rates have been shown to provide sufficient information on TB trends in that particular setting.

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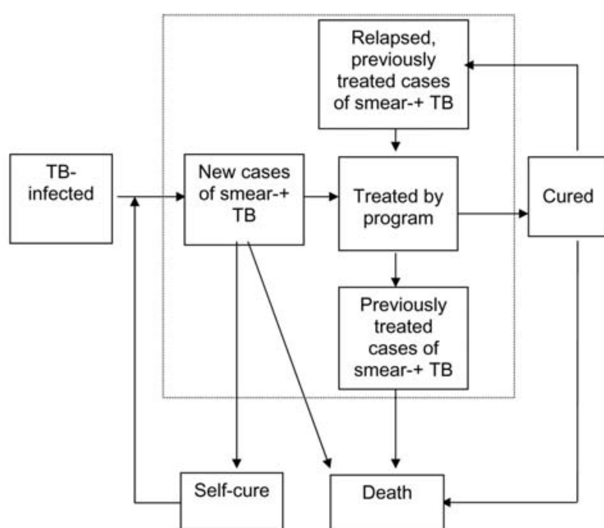
Appendix

Model 1

Model 1, developed by Styblo (1,2), is presented in Appendix Figure 1. The case detection rate in model 1 is not a rate but a ratio: it does not reflect the speed at which cases are detected, but the proportion of incident cases detected. Model 1 assumed that, in the absence of treatment, the duration of the infectious period is 2 years. Each new self-reporting case was assumed to be detected after an average of 4 months. The case detection rate (the proportion of new cases detected) would thus directly determine the prevalence of new smear-positive tuberculosis (TB).



Appendix Figure 1. Model 1, used by Styblo, of tuberculosis case detection and treatment outcome in tuberculosis control program. Prevalent cases are those within dotted line.

Since the interest of this article is to assess case detection, the left part of Figure 1 is concentrated on, which is relevant for the prevalence of new cases of smear-positive TB only (Appendix Figure 2A). When Appendix Figure 2A and the assumptions above are used, the following expressions can be derived:

$$P_{new} = I_{new}\, pyr^{-1} \cdot CDR \cdot 0.33\, pyr + I_{new}\, pyr^{-1} \cdot (1 - CDR) \cdot 2pyr = 2 \cdot I_{new} \cdot (1 - 0.83\, CDR) \quad (1)$$

Where

P_{new} = prevalence ratio of new (i.e., never treated) cases of smear-positive TB

I_{new} = incidence rate (pyr^{-1}) of new smear-positive TB

CDR= case detection rate = proportion of cases detected

By definition:

$$N_{new} = CDR \cdot I_{new}\, (pyr^{-1}) \quad (2)$$

Where

N_{new} = notification rate (pyr^{-1}) of new cases of smear-positive TB

and thus

$$N_{new} / P_{new} = 0.5\, pyr^{-1} \cdot CDR / (1 - 0.83CDR) \quad (3)$$

Model 2

Model 2 was used by Dye et al. and assumes that incident cases are at risk for case detection and for death or self-cure (Appendix Figure 2B) (modified from [3]). A similar approach is used by others (4). If the rates in model 2 were constant (i.e., independent of time since onset of disease), the combined rate of death and self-cure would be 0.5 pyr^{-1} if the average duration of disease were 2 years in the absence of case detection. Indeed, Dye et al. assumed a rate of death of 0.3 pyr^{-1} and a rate of self-cure of 0.2 pyr^{-1} (3). The patient diagnostic rate (PDR) is defined as the rate at which patients are diagnosed. The proportion of incident cases detected (the case detection rate [CDR]) therefore equals:

$$CDR = PDR\, pyr^{-1}/(PDR + 0.5)\, pyr^{-1} \quad (4)$$

Which is equivalent to:

$$PDR\, (pyr^{-1}) = 0.5\, pyr^{-1} \cdot CDR/(1 - CDR) \quad (5)$$

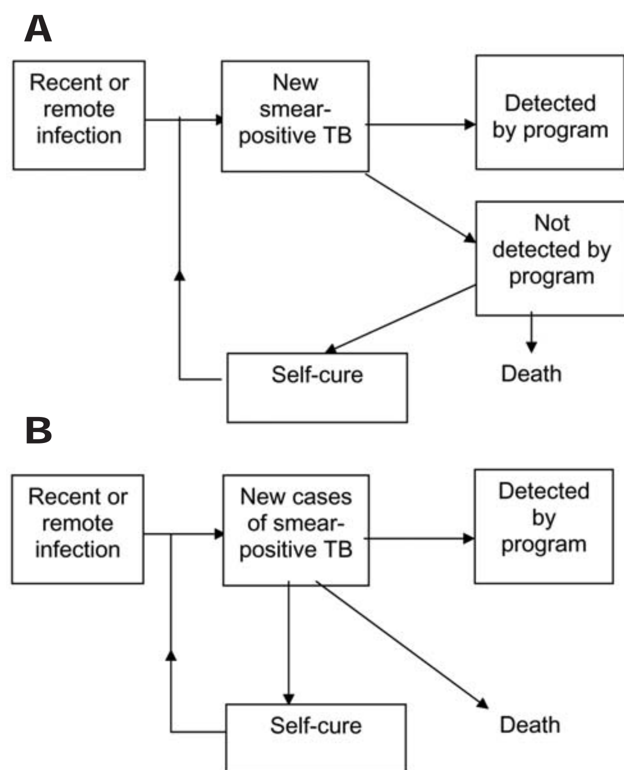
Since PDR may be estimated as N_{new}/P_{new} this can also be presented as:

$$N_{new}/P_{new} = 0.5\, pyr^{-1}\, CDR/(1 - CDR) \quad (6)$$

And since $N_{new} = CDR \cdot I_{new}$:

$$P_{new} = 2\, pyr \cdot I_{new}\, pyr^{-1} \cdot (1 - CDR) \quad (7)$$

To assess to what extent a constant rate of detection (assumed by model 2) is supported by data on delay before diagnosis, we



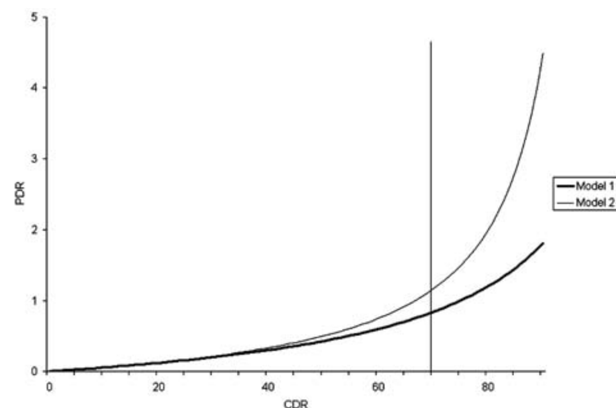
Appendix Figure 2. Models 1 and 2 on tuberculosis case detection. A) the arrows depict proportions of cases moving from one compartment to another. B) Model 2, used by Dye et al. with rate of case detection (PDR). The arrows depict rates.

used data from the Netherlands Tuberculosis Register. From 1996 to 2002, a total of 468 new cases of smear-positive TB were diagnosed among the Dutch; these cases were found through passive case finding and had a recorded delay in treatment. Person-weeks at risk for detection were estimated by week since onset and used as the denominator for the rate of detection. Patient diagnostic rate was first estimated ignoring death rates and self-cure, and then by assuming an average rate of death and self cure of 0.5 pyr^{-1}.

Results

The relationship between case detection rate and patient diagnostic rate according to models 1 and 2 is presented in Appendix Figure 3. In both models, a one-to-one, nonlinear relationship exists between case detection rate and patient diagnostic rate: patient diagnostic rate increases with increasing case detection rates. This increase is steepest in model 2.

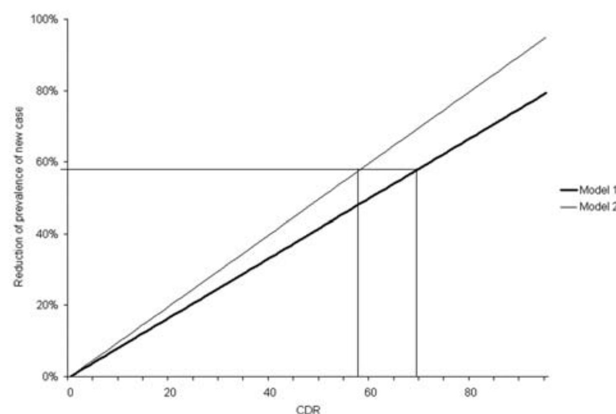
However, the same case detection rate in models 1 and 2 represent different effects on TB prevalence. For instance, a case detection rate of 70% according to model 1 (which is the basis of the current WHO goal) corresponds with a reduction of the prevalence of new cases of smear-positive TB of 58%. According to model 2, to achieve a 58% reduction of this prevalence, a case detection rate of 58% is required (Appendix Figure 4). If the goal



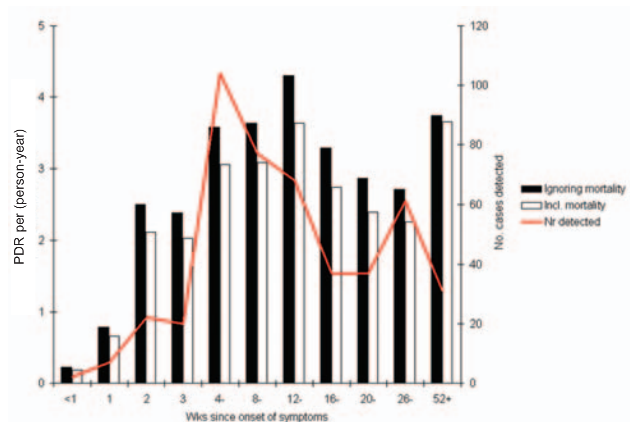
Appendix Figure 3. Relationship of case detection rate (CDR) and patient diagnostic rate (PDR) according to model 1 and model 2.

is to reduce the prevalence of new cases of smear-positive TB by 58%, patient diagnostic rate would need to be 0.84, according to model 1, and 0.69 according to model 2. However, if the case detection rate goal is maintained at 70% while using model 2 (as was done by Dye et al. [3]), the corresponding patient diagnostic rate would be 1.17. Achieving this goal would be associated with a higher effect on TB prevalence than achieving the goal of 0.84 suggested by model 1.

In model 2, the patient diagnostic rate and the combined rate of death and self-cure were assumed to be constant, i.e., independent of time since diagnosis. The rate of detection based on reported patient's and doctor's delay in the Netherlands is presented in Appendix Figure 5. The rates of detection, first by ignoring and then by taking into account death and self-cure, were approximately 3.0 and 2.5 per person-year, respectively. The last figure corresponds with a case detection rate of 84%, according to expression (4). Patient diagnostic rate was lower during the first 4 weeks of disease. During the first 4 weeks, the rate increased approximately linearly from 0 to 2.5 per person-year.



Appendix Figure 4. Reduction of prevalence of new smear-positive tuberculosis depending on the case detection rate (CDR) according to model 1 and model 2.



Appendix Figure 5. Estimates of the patient diagnostic rate (PDR) in the Netherlands, depending on the duration of symptoms.

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Potential Public Health Impact of New Tuberculosis Vaccines

Elad Ziv,* Charles L. Daley,*† and Sally Blower‡

Developing effective tuberculosis (TB) vaccines is a high priority. We use mathematical models to predict the potential public health impact of new TB vaccines in high-incidence countries. We show that preexposure vaccines would be almost twice as effective as postexposure vaccines in reducing the number of new infections. Postexposure vaccines would initially have a substantially greater impact, compared to preexposure vaccines, on reducing the number of new cases of disease. However, the effectiveness of postexposure vaccines would diminish over time, whereas the effectiveness of preexposure vaccines would increase. Thus, after 20 to 30 years, post- or preexposure vaccination campaigns would be almost equally effective in terms of cumulative TB cases prevented. Even widely deployed and highly effective (50%–90% efficacy) pre- or postexposure vaccines would only be able to reduce the number of TB cases by one third. We discuss the health policy implications of our analyses.

Tuberculosis (TB) remains one of the leading causes of illness and death in the world. One third of the world's population is estimated to be infected with *Mycobacterium tuberculosis*, the causative agent of TB (1). This reservoir of infected persons leads to ≈8 million new cases of TB and 2 million deaths each year. Approximately 80% of all new TB cases in the world occur in 22 countries that have incidence rates from 68 to 584 per 100,000 population (2). The priorities for TB control programs in these areas are identifying and treating active cases. Unfortunately, only 40% of smear-positive pulmonary cases are detected globally, and, of these cases, 28% to 80% are treated successfully (2). Most high-incidence countries also use the only available TB vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). Although BCG is the most widely used vaccine in the world, its efficacy in preventing adult forms of TB is relatively poor, with an average efficacy ≈50% (3). A new, more effective vaccine would be expected to improve TB control substantially, and therefore, vaccine development is one of the highest priorities in TB research (4,5). The Gates Foundation recently provided nearly $83 million in grants to boost TB vaccine research (6).

Recent sequencing of the *M. tuberculosis* genome as well as new developments in proteomics and comparative genomics have led to renewed interest in developing new, more effective vaccines against TB (7,8). Vaccines currently under development include subunit vaccines (9), naked DNA vaccines (10,11), and attenuated mycobacteria, including recombinant BCGs expressing immunodominant antigens and cytokines (12). Phase I clinical trials of several of these vaccines are under way or scheduled to begin very soon (13,14). TB vaccines under development can be divided into two categories: preexposure or postexposure vaccines. Preexposure vaccines prevent infection and subsequent disease; these vaccines are given to uninfected persons. Postexposure vaccines aim to prevent or reduce progression to disease; these TB vaccines will be given to persons who are already infected with *M. tuberculosis*. In industrialized countries where TB incidence is low, a preexposure vaccine is the most effective for TB control (15). However, the most effective type of vaccine to control TB epidemics in high-incidence countries, where prevalence of latent TB infection is high, is not apparent. We use mathematical models to predict the potential public health effect of new TB vaccines for epidemic control in high-incidence countries. We evaluate the effect of both pre- and postexposure TB vaccines on two outcome variables: the number of new infections and the number of new cases of disease. We then discuss health policy implications of our analyses.

Prediction Methods

We used mathematical models to compare the potential public health impact of mass vaccination campaigns that used either pre- or postexposure vaccines. We assessed the public health impact in terms of the cumulative percentage of infections prevented and the cumulative percentage of TB cases prevented. We modeled the potential effect of vaccines in developing countries with a high incidence and

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prevalence of infection. Our simulated incidence ranged from 100 to 200 new TB cases per 100,000 persons per year, and we assumed that 28%–50% of the population was latently infected with *M. tuberculosis*. We also assumed that treatment rates were low to moderate (i.e., that 40%–60% of TB patients would be treated and cured). We modeled the potential public health impact of high-efficacy (50%–90%) vaccines and high vaccination coverage rates (60%–90%). We used two separate mathematical models to assess the effect of vaccination: a pre- and a postexposure vaccine model (see Appendix). Our models are similar to those developed by Lietman and Blower (15,16), but we extended them to include the possibility of reinfection of latently infected persons. We analyzed both of our models with uncertainty and sensitivity analysis based on Monte Carlo methods (17–20) (see Appendix for further details) to quantify the effect of vaccine efficacy, duration of vaccine-induced immunity, and vaccination coverage rates on the cumulative percentage of infections and TB cases prevented.

Both our vaccine models reflect the basic pathogenesis of TB (Figures 1 and 2), as in our previous models (21–27). When persons become infected with *M. tuberculosis*, one of the following can occur: 1) they can progress quickly to disease (with probability p); 2) they can become latently infected with *M. tuberculosis* (with probability $1 - p$), and disease never develops; or 3) they can become latently infected with *M. tuberculosis* (with probability $1 - p$) and slowly progress to disease (at rate v). Latently infected persons can also become reinfected (with a relative risk of θ) with a new strain of *M. tuberculosis*. We assessed the potential public health impact of 1,000 different postexposure and 1,000 different preexposure vaccines. Each vaccine had a different efficacy (50%–90%) and average duration of vaccine-induced immunity (10–30 years). We modeled vaccination coverage rates from 60% to 90%. We modeled a mass vaccination campaign at year zero, and then continuous vaccination of each target population each subsequent year.

Our pre- and postexposure vaccine models were designed to vaccinate different populations: preexposure vaccines were designed for uninfected persons, and postexposure vaccines were designed for latently infected persons. We modeled vaccine efficacy for the 1,000 postexposure vaccines by the magnitude of the vaccine's effect on reducing the rate of latently infected persons' progressing to disease (Figure 1). Efficacy of preexposure vaccines is potentially more complex than that of postexposure vaccines, since preexposure vaccines have several potential mechanisms of action. Thus, we assumed that preexposure vaccines could act by three different mechanisms (Figure 2): 1) by reducing the risk for infection in the uninfected, 2) by allowing infection but reducing the probability of fast progression to disease, and 3) by allowing infection but reducing the rate of progression of latent infection to clinical disease. For each of our 1,000 preexposure vaccines, we varied these three potential mechanisms independently from 50% to 90%.

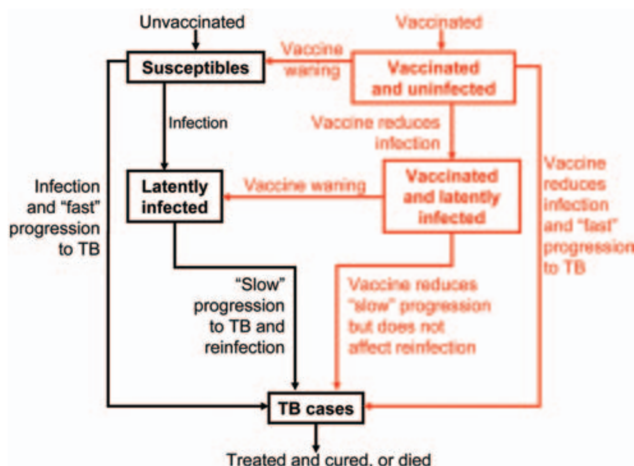

Figure 1. Flow-diagram of postexposure tuberculosis (TB) vaccine model. States and processes that relate to the vaccine are shown in red. Equations are given in the Appendix.

Percentage of Infections and Cases Prevented

In terms of reducing the cumulative number of new infections with *M. tuberculosis*, we found that campaigns that used preexposure vaccines had substantially greater effectiveness than campaigns that used postexposure vaccines (Figure 3A). Preexposure vaccines quickly and substantially reduced the number of new infections; the median cumulative percentage of infections prevented (after 10 years of vaccination) was 46% (interquartile range [IQR] 40%–53%). The effectiveness of preexposure

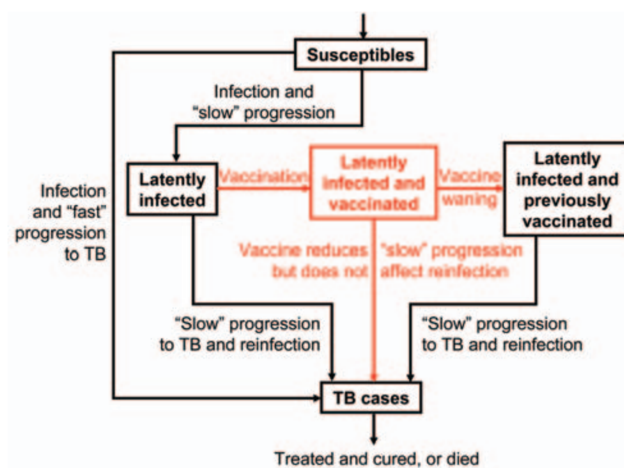

Figure 2. Flow-diagram of preexposure tuberculosis (TB) vaccine model. States and processes that relate to the vaccine are shown in red. Equations are given in the Appendix.

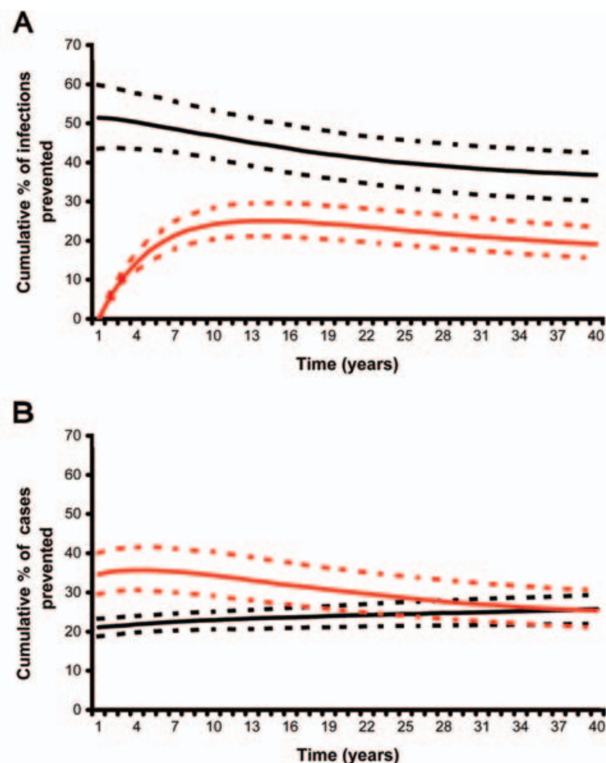

Figure 3. A) Cumulative percentage of new infections with *Mycobacterium tuberculosis* prevented. B) Cumulative percentage of tuberculosis cases prevented. Predictions made using either the preexposure (black lines) or postexposure (red lines) vaccine models and uncertainty analysis.

vaccines in preventing new infections diminished over several decades but remained fairly high. Postexposure vaccines had a considerably slower and smaller effect on reducing the number of new infections; the cumulative percentage of infections prevented rose from 0% (when mass vaccination began) and peaked after ≈10 years at a median of 25% (IQR 21%–29%) (Figure 3A). After 10 years, the effectiveness of postexposure vaccines in preventing new infections gradually declined.

In contrast, in terms of reducing the cumulative number of new cases of TB, postexposure vaccines initially had substantially greater effectiveness than preexposure vaccines. After 10 years of vaccination, postexposure vaccines had reduced the cumulative number of TB cases by a median of 34% (IQR 29%–40%) (Figure 3B); effectiveness diminished slightly over the next few decades, despite continuous vaccination of newly infected latent persons (Figure 3B). Preexposure vaccines, despite having reduced the infection rate by 46% (IQR 40%–53%) (Figure 3A), only reduced the cumulative percentage of TB cases by a median of 23% (IQR 21%–25%) after 10 years (Figure 3B). After 20 to 30 years of continuous vaccination, post- and preexposure vaccines had similar effectiveness in terms of the cumulative percent of TB cases prevented (Figure 3B).

Coverage Rates, Duration of Immunity, and Vaccine Efficacy

To predict the potential public health impact of pre- and postexposure vaccines, in our uncertainty analysis we varied vaccination coverage rates, duration of vaccine-induced immunity, and vaccine efficacy. We determined the quantitative effect of each of these three variables on the cumulative percentage of TB cases prevented by performing a multivariate sensitivity analysis and calculating partial rank correlation coefficients (PRCCs) (Appendix). The cumulative percentage of TB cases prevented increased substantially (PRCC = 0.93, 0.96) as vaccination coverage rates increased from 60% to 90% (Figure 4A, unadjusted data after 20 years of continuous vaccination); this effect was greater for postexposure vaccines than preexposure vaccines. The cumulative percentage of TB cases prevented also increased substantially (PRCC = 0.95, 0.97) as the average duration of vaccine-induced immunity increased from 10 to 30 years (Figure 4B, unadjusted data after 20 years of continuous vaccination); this effect was greater for postexposure vaccines than preexposure vaccines.

We assessed the effectiveness of 1,000 postexposure vaccines that varied in efficacy from 50% to 90%; vaccine efficacy was defined by the degree of reduction in the disease progression rate of latently infected persons. The cumulative percentage of TB cases prevented increased substantially as the postexposure vaccine efficacy increased from 50% to 90% (Figure 4C, unadjusted data after 20 years of continuous vaccination, PRCC = 0.97). Efficacy of preexposure vaccines is more complex than that of postexposure vaccines; therefore, we modeled the efficacy of preexposure vaccines by three different mechanisms (Figure 2) and evaluated the effect of each of the three mechanisms on the cumulative percentage of TB cases prevented. We assumed that preexposure vaccines could reduce the risk for infection in the uninfected (mechanism 1), allow infection but reduce the probability of rapidly progressing to disease (mechanism 2), and allow infection but reduce the rate of progression of latently infections to disease (mechanism 3). We varied each of these three potential mechanisms independently to vary efficacy levels from 50% to 90%. Preexposure vaccines that operated by using mechanism 3 were not effective (PRCC < 0.5) at preventing a substantial cumulative percentage of TB cases, even if these type of preexposure vaccines had a high efficacy. Preexposure vaccines that operated by either mechanism 1 or 2 were effective in preventing TB cases; with preexposure vaccines that operated by reducing the risk of infection in the uninfected (i.e.,

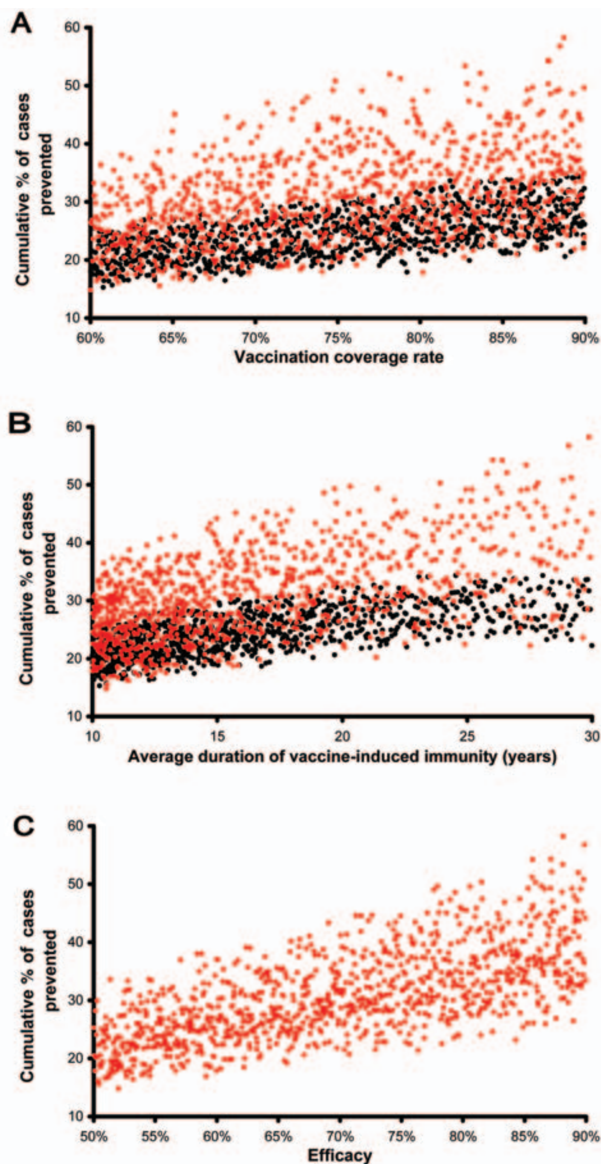

Figure 4. Unadjusted predicted data are plotted; red points represent postexposure vaccines, black points represent preexposure vaccines. A) Cumulative percentage of tuberculosis (TB) cases prevented. B) Cumulative percentage of TB cases prevented. C) Cumulative percentage of TB cases prevented. Cases prevented after 20 years of vaccination are shown as a function of vaccination coverage rates, duration of vaccine-induced immunity, or vaccine efficacy.

mechanism 1) being more effective (PRCC = 0.84) than vaccines that operated by allowing infection but reducing the probability of fast progression to disease (i.e., mechanism 2) (PRCC = 0.66).

Public Health Policy Implications

We evaluated the potential effectiveness of a variety of pre- and postexposure vaccines in controlling TB epidemics in countries that have both a high incidence of disease and a high prevalence of infection. Under these epidemiologic conditions, we found that preexposure vaccines would be almost twice as effective as postexposure vaccines in reducing the infection rate. In contrast, vaccination campaigns that used postexposure vaccines would initially have a substantially greater effect reducing the number of TB cases than campaigns that used preexposure vaccines. However, our predictions show that (despite continuous vaccination) the effectiveness of campaigns using postexposure vaccines would diminish over time but that the effectiveness of campaigns using preexposure vaccines would increase. Hence, after 20 to 30 years, campaigns using either postexposure or preexposure vaccines would be equally effective (because of the complexity of the vaccine mechanisms that we modeled) in terms of the cumulative number of TB cases prevented.

Since preventing disease is more important than preventing infection and to have an immediate, substantial decrease in TB cases is desirable, our results imply that postexposure vaccines would be more beneficial than preexposure vaccines. Our results show that public health officials should expect campaigns that use postexposure vaccines to first appear highly effective, but that effectiveness will decrease with time. We have also shown that the incidence of disease is likely to remain high even if highly effective vaccines that induce long-term immunity are developed and widely deployed. We found that even widely deployed high-efficacy (50%–90%) pre- or postexposure vaccines are only likely to reduce the number of TB cases by one third. Reductions in the number of TB cases would directly translate into reductions in TB deaths (results not shown). Currently the annual TB death rate is 2 million; hence, our results indicate that the type of vaccines we modeled could save ≈700,000 lives per year. These vaccines could also substantially reduce the emergence of drug-resistant TB (22,24).

To understand why even high-efficacy (50%–90%) vaccines are only capable of reducing the TB death rate by one third, how the natural history of *M. tuberculosis* infection differs from other, more "simple," pathogens (e.g., influenza, measles, and smallpox) needs to be examined. For "simple" pathogens, preexposure vaccines can be very effective in reducing epidemic severity because the incidence of disease is a direct function of the incidence of infection. For a "simple" pathogen, if a vaccine reduces infection rates by 80%, then the vaccine will also reduce disease rates by 80%. However, the natural history TB is more complex: the incidence of disease does not directly reflect the incidence of infection with *M. tuberculosis.* The incidence of disease is driven by two sources: susceptible persons who become infected and quickly progress to disease (source 1) and latently infected persons who slowly

progress to disease, often many years after the initial infection (source 2). Both sources make a substantial contribution to the incidence of disease. Preexposure vaccines (given to uninfected persons) will act mainly on reducing the contribution of source 1 to the incidence, but they will have little direct effect on reducing the contribution of source 2. In contrast, postexposure vaccines (given to latently infected persons) will act mainly on reducing the contribution of source 2 to the incidence but will have relatively little effect reducing the contribution of source 1. Therefore, even if highly effective pre- or postexposure vaccines are widely deployed, the incidence of TB in developing countries (as our results show) is likely to remain high.

Also, the increasing HIV epidemic will lead to continuous increases in the incidence of TB in developing countries (26). Currently, what effect co-infection with HIV will have on TB vaccine effectiveness is unclear; possibly, HIV co-infection could reduce vaccine effectiveness. Thus, any new TB vaccine should be evaluated in clinical trials to determine the effect of HIV coinfection on vaccine effectiveness. To reduce the severity of TB epidemics, we recommend that developing and deploying vaccines that act as both pre- and postexposure vaccines are necessary to simultaneously attack both sources that drive the TB rate. Additionally, maintaining high rates of detection and treatment of tuberculosis is necessary, as recommended by the World Health Organization (2); by combining treatment and vaccination strategies, eradicating TB epidemics may be possible, as we have previously shown (16).

Our results have implications for designing both TB vaccines and vaccination campaigns. Highly effective vaccines will be needed to have the public health impact that we have shown (i.e., to reduce the TB death rate by one third). Whether or not the vaccines currently in development will afford this level of efficacy remains to be seen. Moreover, vaccines will need to provide very long-lasting immunity; our current analysis examines the effect of fairly long-lasting vaccines (10–30 years average duration of immunity). Different types of vaccines have different durations of immunity. For example, DNA vaccines should provide lifelong immunity, whereas subunit vaccines will likely require booster vaccinations (28), an approach that would be more logistically difficult and expensive. Also, we have shown that preexposure vaccines are best if they prevent infection (mechanism 1) rather than allow infection but reduce the probability of fast progression to disease (mechanism 2) or reduce the rate of progression of latently infections to disease (mechanism 3). Whether or not new TB vaccines will prevent infection from occurring is not known, but BCG is clearly not able to prevent infection, and vaccines currently in development will likely not be able to do so either (29). As new TB vaccines and other control strategies become available, their potential benefits to TB control efforts can be evaluated by mathematical modeling. Mathematical models can be used as health policy tools to evaluate strategies for controlling TB (30–35); mathematical models also provide insights for predicting the potential public health impact of imperfect HIV vaccines (36–39). Our results show that, because of the complex pathogenic process of TB, high-incidence epidemics are unlikely to be substantially reduced by widely deploying highly effective preexposure or postexposure vaccines. We suggest that to achieve global control of TB, developing a single TB vaccine that functions as both a pre- and a postexposure vaccine is necessary.

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Dr. Ziv is an assistant professor of medicine at the University of California, San Francisco. His previous work on tuberculosis includes a model of the population-level efficacy of targeted prevention for early latent infection.

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Appendix

Vaccine Models

Preexposure Model

Our preexposure vaccine model consists of six ordinary differential equations (1–6) that track the temporal dynamics of persons in six different states: uninfected unvaccinated (X), vaccinated uninfected (X_v), unvaccinated latently infected (L), vaccinated latently infected (L_v), active tuberculosis (TB) (T), and treated and cured (R). The model is given below:

$$dX/dt = (1 - c)\pi - \beta XT - \mu X + \omega X_v \quad (1)$$

$$dX_v/dt = c\pi - \varepsilon_1 \beta XT - (\mu + \omega)X_v \quad (2)$$

$$dL/dt = (1 - p)\beta X_v T - (\nu + \mu + \theta p \beta T)L + \omega L_v \quad (3)$$

$$dL_v/dt = (1 - \varepsilon_2 p)\, \varepsilon_1 \beta XT - (\varepsilon_3 \nu + \mu + \theta p \beta T + \omega)L_v \quad (4)$$

$$dT/dt = p\beta XT + \varepsilon_1 \varepsilon_2 p \beta X_v T + \omega L + \varepsilon_3 \omega L_v + \theta p \beta T (L + L_v) - (\mu + \mu_T + \phi)T \quad (5)$$

$$dR/dt = \phi T - \mu R \quad (6)$$

Persons enter the population at rate π, and a fraction c of them are vaccinated. Uninfected-unvaccinated persons (X) are infected at rate $\beta T(t)$, and then either progress to active disease (T) immediately after infection with probability p, or progress to latent infection with probability $1 - p$. Latently infected persons (L) progress to active disease because of reactivation of latent infection at rate ν. In addition, latently infected persons (L) can also be reinfected at a rate $\beta T(t)$ and progress to active disease with probability p (the probability of rapid progression for newly infected persons) multiplied by the protection afforded by prior infection from rapid progression (θ). Uninfected vaccinated persons (X_v) are protected from infection by probability ε_1. Vaccinated persons

who become infected (L_v) are protected from rapid progression to active disease by probability ε_2. We assume that the vaccine may offer some protection from reactivation (ε_3). The average duration of vaccine-induced immunity is $1/\omega$. The average life expectancy is $1/\mu$. Persons with active TB either die at a rate μ_T or receive effective treatment at a rate ϕ, which leads to recovery (R).

Postexposure Vaccine Model

Our postexposure vaccine consists of six ordinary differential equations (7–12) that track the temporal dynamics of persons in six different states: uninfected (X), unvaccinated latently infected (L), vaccinated latently infected (L_V), previously vaccinated latently infected who have lost immunity (L_W), active disease (T), and treated and recovered (R). The model is given below:

$$dX/dt = (1 - c)\pi - \beta XT - \mu X \quad (7)$$

$$dL/dt = (1 - p)\,\beta XT - (\nu + \mu + \theta p \beta T + \chi)L \quad (8)$$

$$dL_v/dt = \chi L - (\varepsilon\nu + \mu + \theta p \beta T + \omega)L \quad (9)$$

$$dL_w/dt = \omega L_v - (\nu + \mu + \theta p \beta T) L_w \quad (10)$$

$$dT/dt = p\beta XT + \omega(L + L_w) + \varepsilon_3 \omega L_v + \theta p \beta T (L + L_v + L_w) - (\mu + \mu_T + \phi)T \quad (11)$$

$$dR/dt = \phi T - \mu R \quad (12)$$

Persons enter the population at rate π. They become infected at rate $\beta T(t)$ and then either progress rapidly to active disease with probability p or progress to latent infection (L) with probability $1 - p$. Latently infected persons (L) may progress to active disease at rate ν or become reinfected at rate $\theta p \beta T$, where θ defines the protection from reinfection because of natural immunity. Latently infected persons may also be vaccinated. The rate of vaccination is set so that the fraction of latently infected persons who have been vaccinated is equal to c. Latently infected persons who have lost immunity have the same probability of reactivation and disease from new infection as uninfected persons. The average life expectancy is $1/\mu$. Persons with active TB either die at rate μ_T or receive effective treatment at a rate ϕ, which leads to recovery.

Uncertainty and Sensitivity Analysis

We analyzed the two vaccine models by using time-dependent uncertainty analysis (1–6) and numerically simulated the models to calculate the cumulative reduction in new infections with *Mycobacterium tuberculosis* and cases of TB. The reduction in new infections and in cases of TB was calculated as the percentage of the cumulative number of new infections or new cases of TB that would have occurred without vaccination (but with treatment). We used probability density functions to specify each parameter in the two models. We then used Latin hypercube sampling, a modified Monte Carlo sampling procedure, to sample all of the probability density functions (ranges are given in the text). To conduct the uncertainty analyses (for each model), we performed 1,000 simulations; full details of the uncertainty analysis methods are given elsewhere (1–8). We modeled the effects of an initial mass vaccination campaign of the target population and then continued vaccinating the target population. To quantify the sensitivity of the outcome variables to each parameter, we calculated a partial rank correlation coefficient between each parameter value and each outcome variable (1–8).

Parameter Estimates

Our biological parameter values for TB were chosen to simulate epidemics in a high-incidence, high-prevalence region. Estimates for μ, p, and μ_t, are previously described (5). We assume that endogenous immunity to disease from reinfection reduces rapid progression from reinfection by 50% to 100%; if protection is 100%, reinfection does not occur.

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Deaths due to Unknown Foodborne Agents

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This study reviews the available evidence on unknown pathogenic agents transmitted in food and examines the methods that have been used to estimate that such agents cause 3,400 deaths per year in the United States. The estimate of deaths was derived from hospital discharge and death certificate data on deaths attributed to gastroenteritis of unknown cause. Fatal illnesses due to unknown foodborne agents do not always involve gastroenteritis, and gastroenteritis may not be accurately diagnosed or reported on hospital charts or death certificates. The death estimate consequently omitted deaths from unknown foodborne agents that do not cause gastroenteritis and likely overstated the number of deaths from agents that cause gastroenteritis. Although the number of deaths from unknown foodborne agents is uncertain, the possible economic cost of these deaths is so large that increased efforts to identify the causal agents are warranted.

Estimates of foodborne disease deaths are subject to uncertainty because the number of deaths caused by unidentified pathogenic agents in the food supply is unknown. These agents may include bacteria, viruses, parasites, toxins, viruslike agents, or prions. In their influential study of foodborne disease in the United States, Mead et al. (1) estimated that unknown foodborne agents caused 3,400 deaths per year, or 65% of the estimated 5,200 annual deaths from foodborne disease. The Mead study indicates that unknown foodborne agents are an important cause of premature death, with an annual toll comparable to that from accidental fires (3,300 deaths) and drownings (3,300 deaths) (2).

Unknown foodborne agents are unlikely to be reported as a cause of death. The Mead study used hospital discharge and death certificate data on deaths attributed to gastroenteritis of unknown cause to indirectly estimate the number of deaths caused by unknown foodborne agents (1). The estimate by the Mead study is the first systematic assessment of deaths from unknown foodborne agents and deserves scrutiny because little is known about such agents. This study examines the available evidence on unknown foodborne agents for the United States and discusses whether the methods used by the Mead study to estimate deaths from unknown foodborne agents are valid and accurate.

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Evidence for Unknown Foodborne Agents

Evidence suggests that unknown agents cause fatal illness. The Unexplained Death and Critical Illness (UNEX) Project detected 35 deaths caused by possible unknown pathogens in previously healthy persons 1–49 years of age in four U.S. surveillance sites from 1995 to 1998 (3). Deaths from unknown foodborne agents that occurred during intensive care for illnesses resembling infections would have met the UNEX case definition. The death rate from possible unknown pathogens in the UNEX surveillance sites was equivalent to approximately 200 deaths per year among U.S. residents 1–49 years of age, without adjusting for the superior health of the surveillance population.

The Mead study cited evidence that unknown agents are transmitted in food, including well-documented outbreaks of distinctive foodborne illness caused by unidentified agents and the high proportion of reported foodborne outbreaks with an undetermined cause (1). Reported outbreaks probably account for only a small proportion of deaths from unknown foodborne agents because most foodborne outbreaks are never recognized or reported (4) and because some deaths from unknown foodborne agents may result from sporadic illness. However, reported outbreaks represent the only direct evidence of deaths attributable to unknown foodborne agents.

Well-documented outbreaks of distinctive foodborne illness caused by unidentified agents are infrequent in the United States and rarely result in death. Only six reports of such outbreaks have appeared in the Morbidity and Mortality Weekly Report (MMWR) since 1990 (5–10). (A seventh report was excluded because testing for viral agents was incomplete [11].) Two of the reports involved agents later identified as seaweed toxin and *Cyclospora cayetanensis*, which was waterborne in the United States but found elsewhere in food (5,7). A third report

concerned Haff disease, which has a case-fatality rate of 1% (9). The other reports described three distinctive illnesses, but two of the illnesses did not lead to any deaths and therefore appeared to be nonfatal (6,8,10). Brainerd diarrhea, the best known example of an illness caused by an unknown foodborne agent, also appears to be nonfatal (12).

More than two thirds of the 2,800 foodborne outbreaks reported from 1993 to 1997 had an undetermined cause (4). However, most of these outbreaks were probably caused by known pathogens that were not detected, particularly viruses. Recent studies that used molecular diagnostic methods suggest that the causal agents were often members of the *Norovirus* genus (13). The 1,900 outbreaks of undetermined cause reported from 1993 to 1997 resulted in only one recorded death (4), a pattern consistent with the low case-fatality rate for norovirus infections.

The Mead Study

Most deaths from unknown foodborne agents are presumably attributed to unknown causes because clinical laboratory tests are unlikely to detect the causal agent. Physicians describe deaths from unknown causes on hospital charts and death certificates by recording the medical conditions that preceded death. Deaths from unknown agents that caused distinctive conditions could be indirectly estimated from the number of unexplained deaths with such conditions. This approach was adopted by the Mead study, which estimated the number of deaths caused by unknown foodborne agents from the number of deaths involving gastroenteritis of unknown cause.

Gastroenteritis is a common clinical feature of enteric infectious diseases. The Mead study obtained data on deaths involving gastroenteritis of unknown cause from two sources, the annual Multiple Cause of Death (multiple cause) files of death certificates (14), and the annual National Hospital Discharge Survey (NHDS) of nonfederal short stay and general hospitals (15). Causes of death reported on the multiple cause files were coded by using the International Classification of Diseases, ninth revision (ICD-9) from 1979 to 1998, and the 10th revision (ICD-10) after 1998 (16,17). Diagnoses reported on the NHDS files were coded by using the ICD-9 Clinical Modification (ICD-9-CM) (18).

Table 1 lists ICD-9, ICD-9-CM, and ICD-10 codes for gastroenteritis of unknown etiology. In ICD-9, conditions described as "diarrhea" or "gastroenteritis" are coded as 558, together with radiation, toxic, and allergic gastroenteritis. Supplemental injury and external cause codes assigned during cause-of-death coding differentiate radiation and toxic gastroenteritis, as well as gastroenteritis attributable to adverse drug reactions (19). In ICD-9-CM, 558 is subdivided into rubrics identifying radiation, toxic, and allergic gastroenteritis, and a new code for diarrhea was added in 1995 (20). In ICD-10, the codes for gastroenteritis of unknown etiology distinguish infectious conditions, noninfectious conditions in neonates, and noninfectious conditions at other ages.

Table 1. International Classification of Diseases (ICD) codes for gastroenteritis of unknown etiology

ICD revision and code	Title
ICD-9	
Valid for cause-of-death coding	
009.0	Infectious colitis, enteritis, and gastroenteritis
009.2	Infectious diarrhea
558[a]	Other noninfectious gastroenteritis and colitis
Invalid for cause-of-death coding	
009.1[b]	Colitis, enteritis, and gastroenteritis of presumed infectious origin
009.3[b]	Diarrhea of presumed infectious origin
ICD-9-CM	
009.0	Infectious colitis, enteritis, and gastroenteritis
009.1	Colitis, enteritis, and gastroenteritis of presumed infectious origin
009.2	Infectious diarrhea
009.3	Diarrhea of presumed infectious origin
558.9[c]	Other and unspecified noninfectious gastroenteritis and colitis
787.91[d]	Other symptoms involving digestive system: diarrhea
ICD-10	
A09	Diarrhea and gastroenteritis of infectious origin[e]
K52.9	Noninfective gastroenteritis and colitis, unspecified
P78.3	Noninfective neonatal diarrhea

[a]Includes radiation, toxic, and allergic gastroenteritis.
[b]Coded as 558 on the 1979–1998 Multiple Cause of Death files.
[c]Included allergic gastroenteritis before 2000.
[d]Code introduced in 1995.
[e]Includes conditions that are presumed to be infectious.

In the Mead study, the number of deaths from unknown foodborne agents was estimated in two steps (1). First, the annual number of deaths from gastroenteritis of unknown cause was determined by averaging estimates from the 1992–1996 NHDS and multiple cause files. Deaths were classified as gastroenteritis of unknown cause if any cause of death was coded as ICD-9 009 or 558 on the multiple cause files, or if any listed diagnosis was coded as ICD-9-CM 009 or 558.9 on the NHDS files. In the second step, the number of deaths from unknown foodborne agents was determined by assuming that the proportion of foodborne deaths among the 5,000 annual deaths from gastroenteritis of unknown cause was the same as the proportion among deaths from known pathogens that are sometimes or always transmitted through food (67%).

The methods used by the Mead study have several shortcomings. First, the definition of gastroenteritis of unknown cause was imprecise. The definition based on ICD-9 codes included radiation, toxic, and drug-induced gastroenteritis that could be identified and excluded by using the supplemental codes assigned during cause-of-death coding. The definition based on ICD-9-CM codes also included drug-induced gastroenteritis that could be identified by supplemental codes, but excluded gastroenteritis of unknown cause that could be identified by using the new code for diarrhea. Table 2 reports modified ICD-9 and ICD-9-CM definitions of gastroenteritis of unknown cause that avoid these limitations.

A second shortcoming of the Mead study was the low reliability of the NHDS estimate of annual deaths from gastroenteritis of unknown cause. Using the modified ICD-9-CM definition of gastroenteritis of unknown cause, the approximate 95% confidence interval (CI) for the NHDS estimate of 6,194 annual deaths from 1992 to 1996 was 1,627–10,761 deaths (20). The wide interval was due in part to the small number of such deaths (averaging 45 per year) in the NHDS sample. Death certificate data also show that 29% of deaths from gastroenteritis of unknown cause occurred in nursing homes or other settings outside the hospitals in the NHDS sample (21), which indicates that the NHDS estimate was not comprehensive.

The most important shortcomings of the Mead study were the two implicit assumptions about gastroenteritis underlying the estimate of deaths from unknown foodborne agents. The first assumption was that fatal illness attributable to unknown foodborne agents always involved gastroenteritis. The second assumption was that gastroenteritis was accurately diagnosed and reported on hospital charts and death certificates. The two assumptions implied that all deaths from unknown foodborne agents and other unknown agents that cause gastroenteritis (but no other deaths) were attributed to gastroenteritis of unknown cause. Both assumptions are questionable as discussed in the following sections.

Gastroenteritis Caused by Unknown Foodborne Agents

Fatal illnesses attributable to unknown foodborne agents do not always involve gastroenteritis, contrary to the first implicit assumption by the Mead study. Gastroenteritis is not a clinical feature of the only two illnesses caused by unknown foodborne agents known to be fatal, Haff disease and an illness associated with a batch of Kombucha tea (8,9). In addition, most deaths from possible unknown pathogens identified by the UNEX project involved cardiac, respiratory, or neurologic syndromes rather than gastroenteritis, although the initial symptoms (which may have included gastroenteritis) were not reported (3).

The estimate of deaths from unknown foodborne agents by the Mead study omitted deaths from unknown foodborne agents that do not cause gastroenteritis. The number of such deaths is unknown. However, some known foodborne pathogens cause fatal illnesses that do not involve gastroenteritis. In fact, 51% of the estimated deaths from known foodborne pathogens were attributable to four

Table 2. Modified ICD-9 and ICD-9-CM definitions of deaths attributable to gastroenteritis of unknown etiology

ICD-9	ICD-9-CM
Any death with an ICD-9 009.0, 009.2, or 558 code, excluding 1) radiation gastroenteritis deaths, identified by a 558 code plus the supplemental code for an abnormal reaction to a radiological procedure or therapy (E879.2)[a] 2) toxic gastroenteritis deaths, identified by a 558 code plus any supplemental code for poisoning (909.0, 909.1, 960.0-989.9, E850.0-E869.9, E950.0-E950.9, E980.0-E980.9)[a] 3) drug-induced diarrhea deaths, identified by a 558 code plus any supplemental code for an adverse drug reaction (E930.0-E949.9), or by a 009.0 or 009.2 code plus any supplemental code for an adverse reaction to an antibiotic (E930.0-E931.0)[a]	Any death with an ICD-9-CM 009.0-009.3, 558.9, or 787.91 code, excluding 1) drug-induced diarrhea deaths, identified by a 558.9 or 787.91 code plus any supplemental code for an adverse drug reaction (E930.0-E949.9), or else by a 009.0-009.3 code plus any supplemental code for an adverse reaction to an antibiotic (E930.0-E931.0)[b]

[a]The supplemental code must be listed on the same line or one line after the code for gastroenteritis of unknown etiology on part I of the death certificate (for conditions resulting in death), or else both codes must be listed together on part II of the death certificate (for other contributing conditions).
[b]Supplemental codes on NHDS files are not explicitly associated with other diagnoses. Therefore, gastroenteritis of unknown etiology was assumed to be drug-induced if any other diagnosis was one of the specified adverse drug reactions.

agents (*Listeria monocytogenes*, *Toxoplasma gondii*, hepatitis A virus, and *Vibrio vulnificus*) that cause fatal illnesses unaccompanied by gastroenteritis (1). If unknown foodborne agents were as likely as known foodborne pathogens to cause gastroenteritis, then only one half of the deaths caused by unknown foodborne agents would involve gastroenteritis.

Gastroenteritis Diagnosis and Reporting

Gastroenteritis is not always accurately diagnosed or reported, contrary to the second implicit assumption by the Mead study. Physicians do not always recognize known causes of gastroenteritis and may not report the specific cause on hospital charts or death certificates even when it has been identified. As a result, some instances of gastroenteritis attributable to known causes were probably coded as gastroenteritis of unknown origin in hospital discharge and mortality databases.

Infectious causes of gastroenteritis may escape detection because of imperfect testing procedures. Many clinical laboratories do not routinely test stool specimens for some enteric pathogens, including *Escherichia coli* O157:H7 (22), and do not have reliable tests for other enterohemorrhagic *E. coli* serotypes (23). Reliable tests for pathogens like *Campylobacter* may be compromised by inappropriate specimen collection or preparation procedures (24) or by antimicrobial therapy before stool collection. Deaths from undetected pathogens that cause gastroenteritis might consequently be attributed to gastroenteritis of unknown etiology. The UNEX project found that 15% of deaths that were initially unexplained by routine clinical testing were actually attributable to known pathogens, confirming that known pathogens are not always detected (3).

Noninfectious causes of gastroenteritis may also escape detection, notably drug-induced diarrhea, which has been associated with >700 different drugs, including antimicrobial agents (25). Diarrhea occurs in 2% to 25% of patients receiving antimicrobial drugs, depending on the drug (26). Approximately 10% to 20% of antimicrobial drug–associated diarrhea is attributable to opportunistic *Clostridium difficile* infections, but the etiologic agent in other cases is often unknown (26).

Physicians do not always recognize that many drugs besides antimicrobial agents can also cause diarrhea (27). Identifying other drugs as the cause of diarrhea may be difficult when a delay occurs between the start of drug therapy and the onset of diarrhea (25) or when patients are taking many drugs concurrently.

Many deaths occur among patients with drug-induced diarrhea, who may be receiving medications for systemic infections or other serious conditions. For example, hospital patients with antimicrobial drug–associated diarrhea attributable to *C. difficile* infections have a death rate of 4% to 10% (28). If the cause of drug-induced diarrhea was undetermined at the time of death, physicians may list diarrhea on hospital charts or death certificates without specifying a cause. These conditions will be coded as gastroenteritis of unknown etiology in hospital discharge or mortality databases.

Other noninfectious causes of gastroenteritis may not be promptly diagnosed, notably celiac disease, an inherited autoimmune disorder that can cause chronic diarrhea. Celiac disease may be as prevalent in the United States as in Europe, where it affects 0.3% to 0.4% of the population (29). On average, affected U.S. adults first received a diagnosis at age 45 after 11 years of symptoms that included diarrhea in 85% of patients (29). The delay in diagnosis is noteworthy because some persons are likely to die from other causes before celiac disease is recognized. Based on the average age at onset and diagnosis, the prevalence rate in Europe, and current U.S. death rates, approximately 250–325 adults with diarrhea attributable to undiagnosed celiac disease are expected to die each year. If diarrhea from undiagnosed celiac disease is reported on hospital charts or death certificates, it will be coded as gastroenteritis of unknown etiology.

Even when known causes of gastroenteritis are recognized, they may not be accurately coded in hospital discharge or mortality databases. Errors in hospital diagnosis coding often result from incomplete descriptions of diagnoses on the face sheet of medical records (30). Errors in mortality coding are frequently due to inaccurate completion of the cause-of-death section on death certificates (31). Recent studies indicate that 37%–41% of death certificates had improperly completed cause-of-death statements, including 18%–22% that listed nonspecific pathologic processes such as pulmonary edema without identifying the specific cause (31,32). The high frequency of this type of error suggests that some diagnosed causes of gastroenteritis are erroneously reported on death certificates when the nonspecific process (gastroenteritis) is listed without mentioning the specific cause and then coded as gastroenteritis of unknown etiology. However, the accuracy of gastroenteritis coding in hospital discharge and mortality databases does not appear to have been investigated.

Some evidence suggests that infectious causes of gastroenteritis are underreported on death certificates, presumably because the cause was either not detected or not recorded. Table 3 compares the average annual number of reported deaths from enteric infections that cause gastroenteritis with the number estimated by Mead et al. (1) from other sources. An estimated 1,333 deaths occurred, but only 110 (95% CI = 90–131) were reported. The discrepancy implies that approximately 1,200 annual fatal infections were not reported, including 500 *Salmonella*

Table 3. Estimated and reported annual deaths from enteric infections that cause gastroenteritis, United States

Cause (ICD-10 code)	Estimated annual deaths[a]	Average annual reported deaths, 1999–2000 (95% CI)[b]
Salmonella, nontyphoidal (A02.0–A02.9)	582	59.0 (44.9–76.1)
Norovirus (A08.1)	310	0.0 (0.0–3.7)
Campylobacter spp. (A04.5)	124	4.0 (1.1–10.2)
Enterohemorrhagic *Escherichia coli* (A04.3)	91	0.0 (0.0–3.7)
Shigella spp. (A03.0–A03.9)	70	11.5 (6.2-19.7)
Cryptosporidium parvum (A07.2)	66	12.5 (6.9–21.0)
Rotavirus (A08.0)	30	6.0 (2.2–13.1)
Vibrio parahemolyticus (A05.3)	20	1.5 (0.2–5.6)
Brucella spp. (A023.0-A023.9)	11	1.5 (0.2–5.6)
Giardia lamblia (A07.1)	10	2.0 (0.2–7.2)
Clostridium perfringens (A05.2)	7	4.5 (1.6–10.2)
Botulism, foodborne (A05.1)	4	4.5 (1.6–10.2)
Salmonella typhi (A01.0)	3	1.5 (0.2–5.6)
Yersinia enterocolitica (A04.6)	3	0.5 (0.0–3.7)
Staphylococcus food poisoning (A05.0)	2	1.5 (0.2–5.6)
Total	1,333	110.5 (89.9–131.3)

[a]Estimates from Mead et al. (1).
[b]Deaths with any mention of specified cause on the 1999–2000 Multiple Cause of Death files. Causes of death on the 1999–2000 files were coded by using ICD-10, which provides more detailed codes for enteric infections than ICD-9. The 95% confidence interval (CI) measures random variation in the number of deaths, which was assumed to follow a Poisson distribution for <100 deaths and a binomial distribution for ≥100 deaths.

infections, 300 *Norovirus* infections, 100 enterohemorrhagic *E. coli* infections, and 100 *Campylobacter* infections. Some of the unreported infections may have been coded as gastroenteritis of unknown etiology, depending on how the infections were described on death certificates.

Deaths from Gastroenteritis of Unknown Cause

Some cases of gastroenteritis attributable to known causes are likely to be erroneously coded as gastroenteritis of unknown etiology in hospital discharge and mortality databases. Therefore, this study examined deaths attributed to gastroenteritis of unknown cause to determine whether any of the deaths may have involved known causes of gastroenteritis. Data were obtained from the 1994–1998 multiple-cause files, which provide more comprehensive and reliable estimates than the NHDS, using the modified ICD-9 definition of gastroenteritis of unknown etiology (Table 2). Data from more recent years were not examined because the ICD-10 codes used on later files are not fully comparable with the ICD-9 codes used on earlier files (33).

The average annual number of deaths attributed to gastroenteritis of unknown cause from 1994 to 1998 was 4,383 (Table 4), 12% lower than the estimate by the Mead study, which relied in part on NHDS data and included deaths from radiation, toxic, and drug-induced gastroenteritis. The characteristics of deaths attributed to gastroenteritis of unknown cause have been described in detail elsewhere (21). Characteristics potentially indicative of known causes of gastroenteritis include certain other causes of death and the large number of deaths in nursing homes.

Several causes of death reported on death certificates in conjunction with gastroenteritis of unknown cause suggest a possible cause for gastroenteritis. Approximately 1% of deaths had an ICD-9 code for an enteric infectious disease, which implies that physicians had erroneously listed gastroenteritis as a separate cause of death (Table 4). Similar proportions of deaths had codes for bacterial pneumonia or septicemia of known cause, conditions usually treated with antimicrobial drugs. These deaths may have involved simultaneous infections by known and unknown agents, but a more likely explanation is that antimicrobial drug–associated diarrhea occurred and was reported without specifying the cause.

Other causes of death might be indicative of fatal infections by known pathogens that were not accurately diagnosed or reported. Nearly 17% of deaths attributed to gastroenteritis of unknown cause were accompanied by septicemia of unknown cause (Table 4). Some of these deaths may have involved unreported *Salmonella* infections because this agent can also invade the bloodstream. Approximately 19% of deaths were associated with one or more conditions that could have been caused by unreported infections by Shiga toxin–producing pathogens such as the enterohemorrhagic *E. coli*. These conditions included gastrointestinal hemorrhage and vascular insufficiency of the intestine, which resemble hemorrhagic colitis, and renal failure and anemia, both elements of the hemolytic uremic syndrome. Nearly 11% of deaths involved pneumonia of unknown cause and may have included some unreported cases of Legionnaires' disease, which often involves diarrhea. Legionnaires' disease causes an estimated 800–2,700 deaths per year (34), but only 110 deaths per

Table 4. Deaths attributed to gastroenteritis of unknown cause, United States, 1994–1998

Characteristic	Average annual no. of deaths (%)[a]
Total	4,383 (100.0)
Selected other causes of death reported on death certificate (ICD-9 code)	
Enteric infectious diseases (001.0–008.8)	58 (1.3)
Septicemia of known etiology (038.0–038.8)	81 (1.8)
Bacterial pneumonia (481–482.9)	28 (0.6)
Septicemia of unknown etiology (038.9)	726 (16.6)
Hemorrhage of gastrointestinal tract, unspecified (578.9)	167 (3.8)
Acute or unspecified vascular insufficiency of intestine (557.0, 557.9)	136 (3.1)
Anemia, unspecified (285.9)	183 (4.2)
Acute or unspecified renal failure (584.9, 586)	443 (10.1)
Pneumonia of unknown etiology (485, 486)	463 (10.6)
Place of death	
Medical facility	3,058 (69.8)
Nursing home	766 (17.5)
Residence	476 (10.9)
Other, unknown	83 (1.9)

[a]Deaths with any mention of specified cause on the1994–1998 Multiple Cause of Death files.

year from this condition were reported on death certificates from 1999 to 2000.

Nursing home residents accounted for 17.5% of the deaths from gastroenteritis of unknown cause from 1994 to 1998 (Table 4) and had the highest death rate from this cause of any group (21). The nursing home population is at risk from known enteric pathogens and drug-induced diarrhea, which possibly raises the death rate from gastroenteritis of unknown cause because these causes of gastroenteritis are not always recognized or reported. Outbreaks of infectious gastroenteritis are common in nursing homes, where the transmission of enteric pathogens among susceptible persons is facilitated by crowding, shared sources of food and water, and high rates of fecal incontinence (35). Although the cause of these outbreaks has often been undetermined, recent evidence suggests that the causal agents were usually noroviruses (36). Nonepidemic gastroenteritis in nursing homes is thought to be attributable primarily to noninfectious causes, including medications (35). Nursing home residents received an average of 6.7 concurrent medications each (37), which raises their risk for drug-induced diarrhea. Gastrointestinal symptoms (excluding bleeding) were the third most common type of adverse drug event in Massachusetts nursing homes (38) and occurred at an annual rate equivalent to 42,000 adverse events in the U.S. nursing home population.

Discussion

No direct evidence indicates that unknown agents transmitted by food are a major cause of premature death in the United States. The lack of evidence is not surprising because most microorganisms resist cultivation on artificial media, and pathogenic agents that are difficult to culture have undoubtedly eluded identification (39). The innovative study of foodborne disease by Mead et al. (1) has increased awareness of the effects of unknown foodborne agents on health. However, their estimate of deaths from unknown foodborne agents depended on accurately estimating deaths from gastroenteritis of unknown cause, a category assumed to include all deaths from unknown foodborne agents. In fact, some unknown foodborne agents do not cause gastroenteritis, and some deaths attributed to gastroenteritis of unknown cause probably involved known causes of gastroenteritis that were either not detected or not reported, including enteric infections, adverse drug reactions, and celiac disease. The estimate of deaths from unknown foodborne agents consequently omitted deaths from unknown foodborne agents that do not cause gastroenteritis and almost certainly overstated the number of deaths from unknown foodborne agents that cause gastroenteritis.

Indirect estimates of deaths caused by unknown foodborne agents based on U.S. hospital discharge records or mortality data like the Mead study are inherently uncertain. Deaths due to unknown agents that cause gastroenteritis cannot be reliably distinguished from deaths due to known causes of gastroenteritis that were not accurately diagnosed or reported. Death certificate data suggest that many deaths attributed to gastroenteritis of unknown cause could have been due to known causes of gastroenteritis such as *Salmonella* infections or drug-induced diarrhea.

The number of deaths from unknown foodborne agents that do not cause gastroenteritis cannot even be indirectly estimated without first determining the clinical characteristics of such deaths. If the clinical characteristics of deaths caused by unknown foodborne agents are analogous, then one-half of the deaths caused by unknown foodborne agents

do not involve gastroenteritis. However, this analogy may not be accurate because two of the known pathogens that cause fatal illnesses unaccompanied by gastroenteritis (*Toxoplasma* and *Listeria*) cause substantially more deaths than the average foodborne pathogen and might be similarly unrepresentative of unknown foodborne agents.

More precise estimates of deaths attributable to unknown agents are likely to result from the ongoing UNEX project (3). Identifying the causal agents and their mode of transmission is the next step. Careful studies using molecular diagnostic techniques as well as epidemiologic methods might be able to determine whether unexplained deaths are associated with previously unknown microorganisms found in certain foods.

An improved estimate of the number of deaths caused by unknown agents transmitted by food could influence food safety policy in the United States. Regulatory decisions by the federal government are increasingly guided by economic cost-benefit analyses, which take into account the value of lives saved by preventing premature deaths. Although no consensus exists on how to value a life, the U.S. Food and Drug Administration currently employs a value of $5 million per life, a figure derived from wage studies (40). Using this value and the death estimate from the Mead study, the annual cost of deaths caused by unknown foodborne agents would be $17 billion. The actual cost of deaths caused by unknown foodborne agents is uncertain because the number of such deaths is not accurately known. The cost of effective measures to prevent deaths from unknown foodborne agents is similarly undetermined. Despite the uncertainty about the benefits and costs of reducing deaths from unknown foodborne agents, the possible economic losses from such deaths are so large that increased efforts to identify unknown foodborne agents appear to be warranted.

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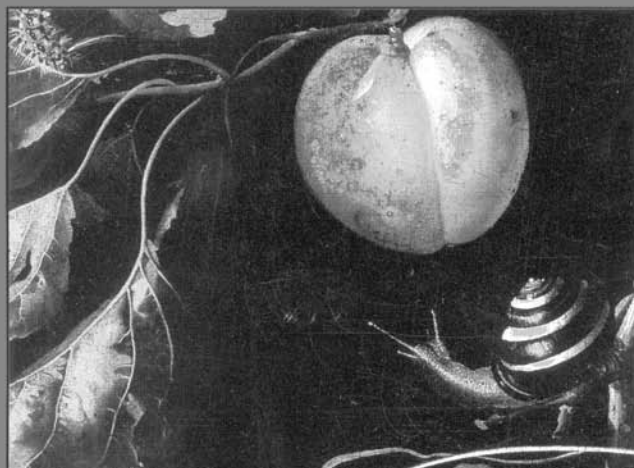

Conference on Emerging Foodborne Pathogens
Pathogen Emergence
Host Susceptibility

Laboratory Exposures to Staphylococcal Enterotoxin B

Janice M. Rusnak,* Mark Kortepeter,* Robert Ulrich,* Mark Poli,* and Ellen Boudreau*

Staphylococcal enterotoxins are 23- to 29-kDa polypeptides in the bacterial superantigen protein family. Clinical symptoms from intoxication with staphylococcal enterotoxins vary by exposure route. Ingestion results in gastrointestinal symptoms, and inhalation results in fever as well as pulmonary and gastrointestinal symptoms. Review of occupational exposures at the U.S. Army Medical Research Institute of Infectious Diseases from 1989 to 2002 showed that three laboratory workers had symptoms after ocular exposure to staphylococcal enterotoxin B (SEB). Conjunctivitis with localized cutaneous swelling occurred in three persons within 1 to 6 hours after exposure to SEB; two of these persons also had gastrointestinal symptoms, which suggests that such symptoms occurred as a result of exposure by an indirect cutaneous or ocular route. Ocular exposures from SEB resulting in conjunctivitis and localized swelling have not previously been reported. Symptoms from these patients and review of clinical symptoms of 16 laboratory-acquired inhalational SEB intoxications may help healthcare workers evaluate and identify SEB exposures in laboratory personnel at risk.

Staphylococcal enterotoxins are 23- to 29-kDa polypeptides in the bacterial superantigen protein family that act by cross-linking HLA-DR or DQ molecules and T-cell receptors. This cross-linking results in potentially pathologic levels of proinflammatory cytokines, such as tumor necrosis factor α, interleukin 2, and interferon-γ (1,2). Therefore, symptoms of mild exposure are anticipated to resemble T-cell–mediated recall responses, similar to a Mantoux skin test.

Staphylococcal enterotoxin B (SEB) is one of at least 15 antigenically distinct enterotoxin proteins (3,4). Clinical symptoms depend on the route of exposure. After inhalation of SEB, clinical features include fever, respiratory complaints (cough, dyspnea, and retrosternal discomfort or chest pain), and gastrointestinal symptoms; severe intoxication results in pulmonary edema, adult respiratory distress syndrome, shock, and death (5,6). Ingesting SEB may cause food poisoning within 1 to 6 hours of exposure, manifested as acute salivation, nausea, and vomiting, followed by abdominal cramps and diarrhea (7,8). As ingesting SEB does not typically result in pulmonary symptoms, gastrointestinal symptoms observed from inhalational intoxication are postulated to result from secondary oral ingestion of SEB concomitant with the inhalational exposure.

One laboratory incident that resulted in nine cases of inhalational intoxication to SEB and several other outbreaks of food poisoning from ingesting staphylococcal enterotoxins have been reported in the literature (5). Symptoms occurring after ocular exposure and localized cutaneous swelling or conjunctivitis from staphylococcal enterotoxins have not been reported. We report three cases of purulent conjunctivitis with localized facial swelling that occurred after ocular exposure to SEB in the laboratory. Two of the three patients also complained of gastrointestinal symptoms. The symptoms in these three mucocutaneous-acquired cases, and summary of symptoms from 16 laboratory-acquired inhalational intoxications with SEB, may help define the clinical spectrum that might be expected after SEB exposures. The full spectrum of clinical signs and symptoms of intoxication with SEB is important to healthcare workers evaluating persons with potential exposures to these agents, including in the context of bioterrorism. This discussion is also relevant to military practitioners, since SEB has been previously developed as an incapacitating biowarfare agent.

Methods

During a review of occupational exposures evaluated in the Special Immunizations Clinic at the U.S. Army Medical Research Institute of Infectious Diseases from 1989 to 2002, clinical evaluations of three laboratory workers with symptoms of conjunctivitis and localized swelling after exposure to SEB were identified. Patient records and occupational exposure summaries were reviewed. Additionally, clinical histories of 16 persons with symptoms after inhalational intoxication with SEB,

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obtained from both that research facility's medical records and occupational exposure reports, were reviewed to summarize the spectrum of symptoms resulting from inhalational exposure to SEB.

Results

Patient 1

While injecting SEB into the endotracheal tube of a rabbit, a 22-year-old male laboratory worker sprayed approximately 150 µg of SEB onto his right glove. Sometime later, he recalled scratching his nose and the area adjacent to his right eye. Three hours after the incident, he noted irritation, pruritus, and a yellow discharge from his right eye. Nine to 12 hours after the incident, he had onset of gastrointestinal symptoms (nausea, abdominal cramps, and loose stools [approximately eight nonbloody loose stools over the next 8 hours]), nasal congestion, postnasal drip, and a self-reported fever. The following morning (approximately 18 h after the incident), he awoke with profound swelling of the right lower eyelid and passed three more loose stools. He did not have headache, chills, vomiting, cough, dyspnea, chest discomfort, or myalgias.

Physical examination was remarkable for diffuse hyperemia of the eye, mildly edematous conjunctiva inferiorly, and edema of the lower lid. The patient was given loperamide for control of diarrhea and sulfacetamide ophthalmic ointment to the right eye four times daily. Gastrointestinal symptoms resolved within 2 days, and the ocular symptoms, nasal congestion, postnasal drip, and febrile symptoms resolved within 4 days. The laboratory worker discontinued loperamide at day 2 and sulfacetamide ointment at day 4.

The amount of SEB in the spray was estimated to be <150 µg. However, the amount of SEB exposure to the right eye was even less, since only a portion of the solution was sprayed onto his hand and rubbed into his eye. The worker was wearing a Tyvek suit (DuPont, Wilmington, DE) and gloves at the time of the exposure but no goggles or respirator. As a consequence, safety measures were modified to recommend only Leur-Lock syringes (Baxter International Inc., Deerfield, IL) and to require eye protection and surgical masks while working with the toxin.

Patient 2

During the reconstitution of SEB within a biosafety cabinet, a 20-year-old laboratory worker injected the contents of a syringe containing 500 µg of SEB into a sealed vial and was exposed when the solution in the vial came under pressure. Approximately 100 µL of SEB in solution foamed from the sealed vial. A small portion of the solution came into contact with the fourth finger on her left hand. She was not wearing gloves. She immediately washed the site with soap and water for 15 seconds and rinsed the soap from her hands. Before she dried her hands, she unconsciously rubbed her left eye with her left hand.

Within 6 to 9 hours of exposure, she had onset of a thick mucous discharge from her left eye, a swollen eye lid, nausea, and loose stools. She had no fever, headache, cough, dyspnea, chest discomfort, vomiting, or myalgias. She was seen at a local emergency room that evening, and was given gentamicin eye drops with a presumed diagnosis of "pink eye" and phenergan for nausea. She was told to remove her contact lenses. The following morning, she was seen in the Special Immunizations Clinic for evaluation for a potential occupational exposure, after reporting to her supervisor that her symptoms might be related to contact with SEB the previous day. Physical examination showed swelling of the left eyelid and discharge from the eye. The discharge was initially described as "long threads" and was subsequently noted to have a yellow color and tear-like appearance. Her symptoms of nausea and diarrhea continued; symptoms exacerbated with food intake. The gastrointestinal symptoms resolved in 3 days, and the ocular symptoms in 5 days.

The estimated syringe loss was 500 µg, but the amount of exposure was estimated to be ≤50 µg, since only a small portion of the solution was in contact with her hand. Because the toxin is extremely water soluble, the immediate washing of the hands should have effectively removed a large amount of the toxin.

Patient 3

One hour after cleaning a dime-size amount of liquid, likely to have been SEB, found outside a biosafety cabinet, a 23-year-old laboratory technician noted bilateral eye irritation, conjunctival erythema, and an excessive yellow ocular discharge that continued throughout the day. She awoke the next morning (day 2) with facial swelling, persistent ocular symptoms, and a subconjunctival hemorrhage (possibly resulting from SEB transfer from hand to eye). She medicated herself with diphenhydramine and brompheniramine and noted improvement in her symptoms the following day (day 3).

On the morning of day 3, she visited the Special Immunizations Clinic for evaluation. At that time, the facial swelling had resolved, and the ocular symptoms had nearly resolved. She had no fever, headache, cough, dyspnea, chest discomfort, nausea, vomiting, diarrhea, or myalgias. Physical examination was remarkable for bilateral conjunctival injection and a 5-mm x 2-mm subconjunctival hemorrhage at the inferolateral margin of the right iris. Complete blood count and erythrocyte sedimentation rate were within normal limits. She was seen by her private ophthalmologist later that day, who recommended no specific treatment. Her symptoms resolved on day 4.

Subsequently, this patient had mild ocular erythema and irritation (no facial swelling or conjunctivitis) while in the laboratory where SEB studies were ongoing. These symptoms resolved immediately after she left the room, which suggests hypersensitivity. She was advised to avoid entering the laboratory when SEB was being used or consider prophylactic use of antihistamines to control symptoms.

Inhalational Cases

Three historical events that occurred during the now disbanded U.S. offensive biologic warfare program resulted in inhalational exposures to SEB and subsequent intoxication. The spectrum of symptoms occurring in the three events is summarized in the Table.

In early 1963, two persons were exposed to SEB as a result of a ruptured hose that contained a crude filtrate of SEB under moderate pressure. Acute asthmatic bronchitis developed in one of these persons within a few hours of exposure. Fever, headache, myalgias, nonproductive cough, dyspnea, nausea, vomiting, and diarrhea developed in both persons, with maximal symptoms at 12 hours and resolution of symptoms by day 3.

In June 1963, five of seven persons became ill within 24 hours of exposure to a highly purified SEB aerosol after a dose-titration experiment in monkeys using a Henderson apparatus for administration of the aerosol; four of the persons required hospitalization (9). The source was suspected to be residual SEB from fur on the monkeys' heads, which had not been wiped after the exposure to SEB. Fever, cough, sternal tightness, anorexia, nausea, and vomiting were prominent features of intoxication; these signs and symptoms started within a few hours to as late as 24 hours after exposure.

The third event occurred in August 1964, when a leak in tubes carrying aerosolized SEB to test monkeys resulted in exposure of 15 persons. Ten persons became symptomatic, and 9 of them were hospitalized (5).

Onset of symptoms from inhalational intoxication was within 1–1/2 hours to 24 hours of exposure (most within

Table. Signs and symptoms of inhalational intoxication to staphylococcal enterotoxin B

Signs and symptoms	Event 1[a] (1963) N = 2	Event 2[b] (1963) N = 4	Event 3 (1964) N = 10	Total (%)
Generalized				
Fever	2	4	9	15/16 (93.7)
Chills	2	2	9	13/16 (81.3)
Headache	2	2	9	13/16 (81.3)
Myalgia	2	1	8	11/16 (68.7)
Fatigue	ND[c]	2	8	10/14 (71.4)
Malaise	ND	2	7	9/14 (64.3)
Lower respiratory				
Cough	2	3	10	15/16 (93.7)
Dyspnea	2	2	4	8/16 (50.0)
Retrosternal or chest pain	ND	3	5	8/14 (57.1)
Wheezing	1	0	1	2/16 (12.5)
Gastrointestinal				
Nausea	2	4	6	12/16 (75.0)
Vomiting	2	3	4	9/16 (56.3)
Anorexia	2	2	5	9/16 (56.3)
Abdominal cramps	ND	1	2	3/14 (21.4)
Diarrhea[d]	2	0	0	2/16 (12.5)
Gas	ND	0	1	1/14 (7.1)
Hepatitis	0	0	1	1/14 (7.1)
Upper respiratory				
Pharyngeal injection	ND	2	3	5/14 (35.7)
Rhinorrhea, postnasal drip, or sinus congestion	ND	2	2	4/14 (28.6)
Sore throat	ND	1	2	3/14 (21.4)
Otitis	ND	1	1	2/14 (14.3)
Hoarseness	ND	0	1	1/14 (7.1)
Other				
Conjunctival injection	ND	2	2	4/14 (28.6)
Burning eyes	ND	0	1	1/14 (7.1)
Flushed face	ND	1	0	1/14 (7.1)

[a]Only occupational summary reports reviewed (medical records not available).
[b]No records available on the one nonhospitalized symptomatic person.
[c]ND, no data.
[d]Loose stools noted in one person in the second and the third events.

12 hours of exposure), and the duration of symptoms was generally 3–5 days. In addition to the previously reported and commonly observed symptoms of fever, headache, myalgias, pulmonary symptoms, and gastrointestinal symptoms, fatigue and malaise were observed in most persons (Table). While diarrhea was reported in a few cases, it was not a prominent finding with SEB intoxication by inhalation. Conjunctival injection, previously reported in the literature, was noted only in four persons.

Newly reported symptoms include upper respiratory symptoms (e.g., sore throat, profuse postnasal drip, sinus congestion, rhinorrhea, coryza, and hoarseness). Pharyngeal injection (five persons) and injected tympanic membranes (two persons) were observed; neither had been previously reported in the literature.

Discussion

Outside the context of food poisoning, few physicians would be expected to have experience evaluating persons with SEB intoxication. However, an increase in laboratory exposures and intoxications with staphylococcal enterotoxins can be expected as additional institutions begin work with them as a result of increased funding for biodefense research. Symptoms of conjunctivitis with periocular or facial swelling, acquired by ocular or cutaneous exposure routes, have not been previously reported in the literature. A historical occupational health Department of Defense report suggests that conjunctivitis and upper respiratory tract symptoms resulting from exposures to staphylococcal enterotoxins were recognized during the time of the U.S. offensive biological warfare program from 1945 to 1969 (10). Therefore, documenting the full clinical spectrum of intoxications with staphylococcal enterotoxins is necessary to educate healthcare workers and safety officers to enable them to identify workers at risk and prevent exposures to staphylococcal enterotoxins.

Clinical symptoms from SEB may vary and are dependent on the dosage and route of exposure (5). While inhaling SEB may result in fever, pulmonary, and gastrointestinal symptoms; ingestion of staphylococcal enterotoxins generally results mainly with gastrointestinal symptoms. The gastrointestinal symptoms noted in two persons with ocular or percutaneous exposures (or both) suggest that gastrointestinal symptoms from SEB may occur by a nonoral route, although transmission of SEB to the gastrointestinal tract via the lacrimal duct cannot be entirely excluded. Also, recurring symptoms of ocular irritation and erythema when in the presence of SEB, and immediate resolution of symptoms when no longer in an SEB area, suggests a possible hypersensitivity to SEB.

The pathophysiology of symptoms from staphylococcal enterotoxins, however, is not fully understood. Staphylococcal enterotoxins are superantigens that act by cross-linking HLA-DR or DQ molecules and T-cell receptors, resulting in high levels of inflammatory cytokines such as interleukin 2, interferon-γ, and tumor necrosis factor (1). Staphylococcal enterotoxins resist inactivation by gastrointestinal proteases; oral dosages as low as 5–20 μg induce emesis in nonhuman primates (4,8). However, nongastrointestinal routes such as intravenous administration of SEB (higher dosages of 20 to 500 μg) also induced emesis in nonhuman primates.

High levels of cytokines alone may cause symptoms similar to SEB intoxication. Cancer patients, given high doses intravenously of the cytokine interleukin-2, have symptoms of fever, malaise, nausea, vomiting, and diarrhea, similar to SE food poisoning (7). Also, intravenous OKT3, a monoclonal antibody used as an immunosuppressant in transplant patients (it binds to T lymphocytes, resulting in early activation of T cells, cytokine release, and subsequent blocking of T-cell functions), has a side effect profile similar to that of SEB—high fever, gastrointestinal symptoms, arthralgias, and pulmonary symptoms (11).

The mechanism of emesis also has been postulated to be related to the stimulation of mast cells and the subsequent release of cysteinyl leukotrienes and histamine (12). L4 171883, a selective inhibitor of LTD4/LTEF receptors, completely eliminated the emetic response and immediate type skin reactions (skin reactions associated with degranulation of cutaneous mast cells) to SEB. Inhibition of prostaglandins by indomethicin or pretreatment with a dual lipoxygenase and cyclo-oxygenase inhibitor (BW 755C) did not prevent emesis or immediate type skin reactions. After degranulation of mast cells, impulses are sent through the vagus and sympathetic nerves to the medullary center, which results in emesis. Severing of the vagus and sympathetic nerves inhibits the emesis response (13).

The mechanism of diarrhea is even less well understood, although it is not by means of activation of adenylate cyclase (5). Histopathologic findings with staphylococcal food poisoning are minimal; they mainly show polymorphonuclear cell infiltrates in the epithelium and lamina propria of the stomach and proximal small intestine (7,8).

SEB intoxication is diagnosed by clinical symptoms and a history of potential exposure to SEB. Definitive diagnosis of inhalational exposure can be made by nasal swabs and induced respiratory secretions for toxin assays, blood and urine for immunoassay, and acute- and convalescent-phase serum, but these tests are not readily available and not reliable for low-dose exposures. While inhalational intoxication with SEB is generally associated with leukocytosis and a mildly elevated erythrocyte sedimentation rate, these findings are nonspecific. Chest x-ray findings of increased interstitial markings, atelectesis, overt pulmonary edema, or acute respiratory distress

syndrome are also nonspecific and only present in inhalational intoxications of SEB (5). Potential changes in serum antibody titers, although relevant, have not been examined.

Toxic and lethal doses of SEB vary greatly between animal species, mostly because of differences in receptor-binding affinities, and also vary depending on the route of exposure (14). In humans, the estimated 50% lethal dose (LD_{50}) is 0.02 µg/kg and 50% effective dose (ED_{50}) is 0.0004 µg/kg by aerosolized exposure (14). No data exist on the LD_{50} and ED_{50} in humans by other routes of exposure. The ED_{50} is estimated to be 0.03–0.26 µg/kg in monkeys and 12–40 µg in chimpanzees, by intraperitoneal or intravenous challenge. The extrapolation of the estimated values of ED_{50} of nonhuman primates to humans would suggest that 2 µg versus 840 µg of SEB would be needed to cause symptoms in a 70-kg person through the ocular or cutaneous route. Occurrence of symptoms in two persons after exposure to dosages of SEB <50 µg provides support that the lower ED_{50} value in monkeys may also apply to humans.

During the offensive biologic warfare program, a contractor report addressing the efficacy of biosafety cabinets noted toxic reactions in persons performing SEB purification studies on open laboratory benches (10). The following symptoms were noted in six persons: conjunctivitis, nondescript chemical irritation of one eye, general skin reaction, severe facial skin reaction, dermatitis, and cold symptoms. Additionally, symptoms mainly of conjunctivitis and acute pharyngitis, but also including vomiting and diarrhea in two cases, were observed in 23 persons wearing surgical masks or face shields while working with SEB. Persons working with SEB within a biosafety cabinet had no symptoms.

As exposure to low dosages of SEB can produce symptoms, these recently reported symptoms have importance both to safety officers and healthcare workers evaluating laboratory workers at risk with potential exposures to staphylococcal enterotoxins. SEB intoxication can mimic an infectious process. The initial diagnosis of the first person who sought medical evaluation in the June 1963 incident was pneumococcal pneumonia; symptoms included acute onset of fever, chills, productive cough, chest pain, and dyspnea. The patient was started on penicillin, which was discontinued after his co-workers exhibited similar symptoms, a finding that supported the diagnosis of SEB intoxication. Even though medical providers had knowledge of SEB exposure in a subsequently hospitalized patient involved in the June 1963 incident, the initial symptoms of this patient still raised concern about a possible infectious cause. That patient was noted to have a flushed face, mild hyperemia of the pharynx, a prominent postnasal drip, a purulent-appearing otitis media and externa without symptoms of ear pain, pulmonary symptoms (productive cough and chest discomfort), and a leukocytosis of 19,500 cells/mm^3. Their differential diagnosis included otitis externa and media, pneumonia, or SEB intoxication. Otic examination was within normal limits 24 hours later, which suggested SEB as the possible cause of the localized swelling. An infectious cause was also considered as the initial primary diagnosis in the initial two patients with conjunctivitis in this series as the cause of the conjunctivitis, gastrointestinal symptoms, or both, with both persons receiving topical ophthalmic antimicrobial agents for conjunctivitis. Healthcare workers evaluating persons who work with SEB need to be aware of the full spectrum of toxicity symptoms associated with SEB to avoid misdiagnosis resulting in unnecessary treatment, to identify breaches in laboratory technique, and to educate persons at risk of the importance of personal protective measures in preventing SEB exposure and intoxication. These cases emphasize that personal protective measures such as biosafety cabinets, gloves, and eye protection are paramount when working with SEB.

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Viral Loads in Clinical Specimens and SARS Manifestations

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A retrospective viral load study was performed on clinical specimens from 154 patients with laboratory-confirmed severe acute respiratory syndrome (SARS); the specimens were prospectively collected during patients' illness. Viral load in nasopharyngeal aspirates (n = 142) from day 10 to day 15 after onset of symptoms was associated with oxygen desaturation, mechanical ventilation, diarrhea, hepatic dysfunction, and death. Serum viral load (n = 53) was associated with oxygen desaturation, mechanical ventilation, and death. Stool viral load (n = 94) was associated with diarrhea, and urine viral load (n = 111) was associated with abnormal urinalysis results. Viral replications at different sites are important in the pathogenesis of clinical and laboratory abnormalities of SARS.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease that affected 8,098 persons and caused 774 deaths from November 1, 2002, to September 26, 2003 (1). A novel coronavirus (SARS-CoV) was consistently isolated from SARS patients in three different continents independently (2–4). Animal models using macaque monkeys, ferrets, and domestic cats were established (5–7); no extrapulmonary lesions could be identified in these animals, although virus isolation and reverse transcription–polymerase chain reaction (RT-PCR) results for viral RNA from pharyngeal secretions, tracheobronchial secretions, urine, rectal swabs or stool, kidney tissue or lung tissues were positive.

Recently, we reported the use of RT-PCR to detect SARS-CoV RNA from nasopharyngeal aspirates (NPA) and throat swab, urine, and stool specimens (8). We also developed quantitative RT-PCR (RT-qPCR) assays using the LightCycler System (Idaho Technology Inc., Idaho Falls, ID) to augment the sensitivity of detection (9). The serial viral load in NPA was also used to monitor clinical progress and response to antiviral therapy, and viral load in serum on admission was used as a marker of prognosis (10–12). Extrapulmonary manifestations, such as hematologic changes, diarrhea, and liver dysfunction are common in SARS patients but not in animal models (13–15). In this study, we assayed and analyzed viral load in clinical specimens taken from different anatomical sites from day 10 to 15 after the onset of symptoms to understand the role of SARS-CoV in the pathogenesis of clinical manifestations and laboratory test abnormalities in SARS patients.

Patients and Methods

We included in this quantitative virologic study 154 patients who fulfilled the modified World Health Organization (WHO) definition of SARS and whose treatment was managed in the United Christian Hospital and Caritas Medical Centre of Hong Kong Special Administration Region of China. All patients' infections were either serologically confirmed (fourfold rise of indirect immunofluorescent antibody titer in serum taken on admission and within 28 days of symptom onset) or RT-PCR was positive for SARS-CoV RNA (confirmed from their clinical specimens for those who died or did not seroconvert before day 28). The case definition includes temperature of ≥38°C, cough or shortness of breath, and new pulmonary infiltrates shown on chest x-ray or high-resolution computed tomographic scan in the absence of an alternative diagnosis to explain the clinical signs and symptoms.

The treatment protocol, clinical manifestations, and progression of disease in part of this cohort had been previously reported (10,11,16). In brief, patients were prospectively monitored for diarrhea, oxygen desaturation,

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mechanical ventilation; laboratory evidence of lymphopenia, renal impairment, liver dysfunction, or abnormal urinalysis during the first 15 days; and death. Diarrhea was defined as bowel movements ≥3 times per day for 2 consecutive days. Oxygen desaturation was defined as <90% oxygen saturation measured by pulse oximetry while breathing room air. Some of these patients later required mechanical ventilation. Hepatic dysfunction was defined as a mean level of alanine aminotransferase (ALT), alkaline phosphatase (ALP), or both, greater than the upper limit of normal from day 10 to day 15 after onset of symptoms. Impaired renal function was defined as a serum creatinine level higher than the reference range on 2 consecutive days. Lymphopenia was defined as an absolute lymphocyte count <1,000/µL on 2 consecutive days. Abnormal urinalysis results were defined as proteinuria (>30 mg/dL), microscopic hematuria (>10 erythrocytes/µL), pyuria (>50 leukocytes/µL) on a dipstick test, or casts in urine (Combur Test, Roche Diagnostics GmbH, Mannheim, Germany) examined with inverted microscope by an experienced technician.

To diagnose SARS-CoV infection, NPA and serum samples were taken on admission. Convalescent-phase serum samples were taken between days 7 and 28 after symptom onset. In all patients, RT-PCR for SARS-CoV RNA was performed on the NPA collected on admission. RT-qPCR was performed for patients who had NPA, serum, stool, and urine specimens collected on days 10 to 15 after the onset of symptoms. All virologic diagnostic laboratory tests, including viral culture, RT-PCR, RT-qPCR, and immunofluorescent antibody detection for immunoglobulin (Ig) G, were performed according to our previously published protocols (2,10). NPA was obtained by suction through both nostrils with a Pennine 6 mucus extractor (Pennine Healthcare, Derby, UK) and mucus specimen trap (MST-7000, Pennine Healthcare). The catheter was connected midway between the tip of the nose and the auditory meatus; it was rotated continuously and slowly retrieved with intermittent suction at a negative pressure of 100 mm Hg for 15 s. The procedure was repeated in the other nostril. Secretions stuck to the lumen of the catheter were transferred to the mucus trap by flushing with 1 mL of viral transport medium, which consists of Earle's Balanced Salt Solution (BioSource International, Camarillo, CA), 4.4% bicarbonate, 5% bovine serum albumin, vancomycin (100 µg/mL), amikacin (30 µg/mL), and nystatin (40 U/mL). A 10% stool suspension was made by swirling approximately 1 g of stool in 10 mL of viral transport medium. Midstream urine was collected in a sterile container. Serum from clotted blood was collected and stored at 4°C for antibody testing and at –20°C before RNA extraction. The other specimens were stored at 4°C before RNA extraction.

RNA from clinical samples was extracted by using the QIAamp virus RNA mini kit (Qiagen, Cologne, Germany) as instructed by the manufacturer. For all specimens, 140 µL of the sample was used for RNA extraction, and extracted RNA was finally eluted in 30 µL of RNase-free water and stored at –20°C. For the RT-qPCR assay, RNA and cDNA were generated as described (9). cDNA was amplified in a 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) by using TaqMan PCR Core Reagent kit (Applied Biosystems). In a typical reaction, 2 µL of cDNA was amplified in a 25-µL reaction containing 0.625 U AmpliTaq Gold polymerase (Applied Biosystems), 2.5 µL of 10x TaqMan buffer A, 0.2 mmol/L of dNTPs, 5.5 mmol/L of $MgCl_2$, 2.5 U of AmpErase UNG, and 1x primers-probe mixture (Assays by Design, Applied Biosystems). The forward primer was 5′-CAGAACGCTGTAGCTTCAAAAATCT-3′ (corresponding to nt 17,718–17,742 of the SARS-CoV genome), and the reverse primer was 5′-TCAGAACCCTGTGATGAATCAACAG-3′ (corresponding to nt 17,761–17,785). The sequence of the reporter probe was 5′-(FAM)TCTGCGTAGGCAATCC(NFQ)-3′ (FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; complementary to nt 17,745–17,760). Reactions were first incubated at 50°C for 2 min, followed by 95°C for 10 min. Reactions were then thermocycled for 40 cycles (95°C for 15 s, 60°C for 1 min). Plasmids containing the target sequences were used as standard controls. To monitor the integrity of RNA extraction for each sample, a housekeeping gene, β-actin, was detected by real-time RT-PCR with two primers: β-actin forward, 5′-CCCAAGGCCAACCGCGAGAAGAT-3′ and reverse, 5′-GTCCCGGCCAGCCAGGTCCAG-3′. All samples were found to contain detectable β-actin RNA.

All timed data were calculated from onset of symptoms. We compared the viral load in these specimens with the presence or absence of diarrhea, oxygen desaturation, mechanical ventilation, lymphopenia, hepatic dysfunction, abnormal urinalysis findings, and death rate by chi-square or Fisher exact test for categorical variables and by Mann-Whitney U test for continuous variables. A two-tailed p value < 0.05 was considered significant. Correlation between the number of anatomic sites with detectable viral load by RT-qPCR and death rate was calculated by linear regression. SPSS (version 11.0, SPSS Inc., Chicago, IL) was used for all analyses.

Results

Of the 154 SARS patients who satisfied the WHO definition for SARS, their age range was 20–80 years (mean 41.5 years). The male-to-female ratio was 0.60, and 31 (20.1%) were healthcare workers. One hundred and fifty patients (97.4%) were ethnic Chinese, and 4 (2.6%) were

Filipino. The following clinical specimens taken from days 10 to 15 from individual patients were available: NPA (n = 142), serum (n =53), stool (n = 94), and urine (n = 111). All patients had laboratory-confirmed SARS, either virologically by qualitative RT-PCR in 116 (75.3%) patients (from 30.5% of NPA, 68.2% of stool samples, and 26.6% of urine samples), by RT-qPCR in 126 (81.8%) patients (from 42.3% of NPA, 41.5% of serum samples, 87.2% of stool samples, and 28.8% of urine samples), or by seroconversion in indirect immunofluorescent antibody assay in 136 (88.3%) patients. Six patients who died before day 28 did not have serologic documentation, and 12 others had not seroconverted by day 28, but all had SARS confirmed by RT-PCR. Viral cultures were positive for SARS-CoV in 20 patients from NPA (n = 18), stool (n = 1), and urine (n = 1).

Besides fever and pulmonary infiltrates shown on x-ray, peripheral blood lymphopenia was the most common hematologic abnormality on admission and afterwards (Table 1). From admission to day 10, the peripheral lymphocyte count in all patients decreased. From days 10 to 19, the peripheral lymphocyte count continued to decrease in patients who ultimately died, whereas in those who survived, lymphocyte count gradually increased (Figure). Seroconversion to SARS-CoV antibodies also started at around day 10. Diarrhea (43.5%) was the most common extrapulmonary clinical manifestation from days 10 to 15, followed by hepatic dysfunction (39.0%). Abnormal urinalysis was found in 87 (56.4%) patients, with increased leukocyte esterase in 49.4%, pyuria in 23%, microscopic hematuria in 20.1%, and proteinuria in 3.8%. None of the patients had impaired renal function from admission to day 20. Oxygen desaturation developed in 73 patients (47.4%) at a mean of 10.2 (standard deviation [SD] = 5.1) days, and 34 patients (22.1%) died from SARS a mean of 32.4 (SD = 10.6) days after onset of symptoms. Only four patients were found to have abnormal mean ALP levels from admission to day 20. Therefore, only the mean ALT level from days 10 to 20 was analyzed for correlation with viral load. The mean ALT level between admission and day 10 was also analyzed. However, no significant association was found between the mean ALT level and the viral loads from different specimens.

Table 1. The dominant clinical features and laboratory abnormalities of 154 patients with SARS[a]

Clinical feature	On admission (%)	Days 10–15 (%)
Oxygen desaturation	2 (1.4)	73 (47.4)
Diarrhea	15 (10.6)	67 (43.5)
Lymphopenia	105 (68.1)	126 (81.8)
Hepatic dysfunction	34 (22.1)	60 (39.0)
Abnormal urinalysis findings	NA	87 (56.4)

[a]SARS, severe acute respiratory syndrome; NA, not applicable.

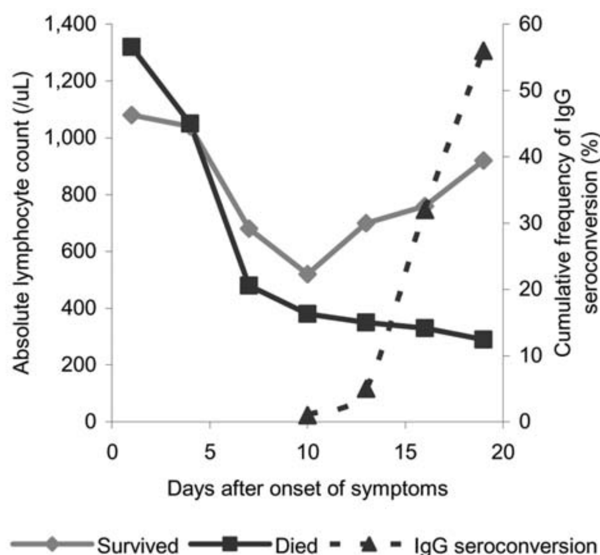

Figure. Serial change of the mean absolute lymphocyte count and immunoglobulin (Ig) G seroconversion of severe acute respiratory syndrome (SARS)–associated coronavirus in 154 SARS patients.

Both the positivity rate (87.2%) and the viral load by RT-qPCR (6.1 $\log_{10}$ copies/mL) were highest for stool specimens, followed by NPA (42.3% and 2.4 $\log_{10}$ copies/mL, respectively). Although virus was more frequently detected in serum (41.5%) than urine (28.8%), their mean viral loads for those specimens were 2.7 $\log_{10}$ copies/mL and 4.4 $\log_{10}$ copies/mL, respectively (Table 2). More patients with lymphopenia on admission had virus detectable by RT-qPCR in NPA ($p = 0.04$, odds ratio [OR] 2.2, 95% confidence interval [CI] 1.7–4.4, data not shown). Both the positivity rate and the viral load in NPA were significantly correlated with diarrhea, oxygen desaturation, mechanical ventilation, hepatic dysfunction, and death rate (all $p < 0.01$) (Tables 3 and 4). A high viral load in serum was also associated with oxygen desaturation, mechanical ventilation, hepatic dysfunction, and death (all $p < 0.01$) but not with diarrhea or abnormal urinalysis findings. Stool viral load was associated with diarrhea, hepatic dysfunction, abnormal urinalysis results, and death (all $p < 0.01$). A higher viral load in urine was significantly correlated with abnormal urinalysis and with diarrhea (both $p < 0.01$). These findings were confirmed by subgroup analysis of 40 patients with specimen samples taken from all four sites (NPA, serum, stool, and urine) (Tables 5 and 6). Again, a detectable viral load in NPA and serum was associated with oxygen desaturation (OR 5.3, 95% CI 1.4–21.1), mechanical ventilation (OR 1.6, 95% CI 1.1–2.4), and death (OR 1.8, 95% CI 1.2–2.7). The stool viral load was associated with diarrhea (OR 18, 95% CI 2.0–16.3), although a higher viral load in urine was no longer associated with abnormal urinalysis results.

Table 2. SARS-CoV and viral load in different clinical specimens taken during days 10 to 15 after the onset of symptoms[a]

Clinical specimens	Mean viral load in log_{10} copies/mL (SD), all specimens	Mean viral load in log_{10} copies/mL (SD), positive specimens only	Positivity by RT-qPCR (%)
NPA (n = 142)	2.4 (3.1)	5.8 (1.7)	60/142 (42.3)
Serum (n = 53)	1.1 (1.4)	2.7 (0.6)	22/53 (41.5)
Stool (n = 94)	6.1 (3.0)	7.0 (2.1)	82/94 (87.2)
Urine (n = 111)	1.3 (2.1)	4.4 (1.3)	32/111 (28.8)

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus; SD, standard deviation; RT-qPCR, quantitative reverse transcription–polymerase chain reaction; NPA, nasopharyngeal aspirates.

In the subgroup analysis of 40 patients who had all types of specimen available for analysis, 34 had antibody seroconversion, and 6 died before day 28 without serologic documentation. Diagnosis of each was confirmed by qualitative RT-PCR. In this group, the number of anatomic sites with positive RT-qPCR strongly correlated with the risk for death (Pearson correlation = 0.517, $p < 0.0005$). No deaths were associated with negative RT-qPCR at all sites or just one positive site. Risk for death was 12.5% with positivity at two sites and 41.7% with positivity at three sites. The death rate increased to 66.7% if RT-qPCR was positive at all four sites.

Discussion

Viral load reflects the dynamic interaction between viral replication and clearance by body defense mechanisms. Examining viral load in SARS patients has been used to diagnose and monitor progress or response to antiviral therapy (10,11). We have shown that viral load in NPA peaked around day 10 (10), with a rapid decrease and concomitant normalization of lymphocyte count and rise in serum antibodies to SARS-CoV (Figure). Presence of virus and viral load in different body fluids may also bear on possible modes of transmission. Infectivity at day 10, reflected by a mean peak viral load of 5.8 log_{10} copies/mL and 7.0 log_{10} copies/mL in positive specimens of NPA and stool, respectively, suggested that respiratory droplets and indirect contact with feces may be important mechanisms of transmission. Viral load has typically been measured in NPA and serum at admission as a diagnostic and prognostic tool (10,12,17). Viral load in body fluids other than NPA and serum has not been studied to elucidate transmission and pathogenesis of SARS.

The importance of SARS-CoV as a respiratory pathogen is supported by the strong association of viral load in NPA with oxygen desaturation, mechanical ventilation, and death. Unexpectedly, viral load in NPA was also associated with diarrhea and hepatic dysfunction. Anecdotal reports of the use of steroids to treat SARS tend to suggest these extrapulmonary manifestations could be

Table 3. Correlation of RT-qPCR of clinical specimens taken during days 10 to 15, and clinical manifestations in 154 patients with SARS[a]

	Diarrhea			O_2 desaturation			Mechanical ventilation			Death		
Specimen	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)
NPA (n = 142)												
RT-qPCR (+) (n = 60)	37	23	< 0.01; 2.5	37	23	< 0.01; 3.1	22	38	< 0.01; 11.3	24	36	< 0.01; 54
RT-qPCR (–) (n = 82)	32	50	(1.3–5.0)	28	54	(1.6–6.2)	4	78	(3.6–35.1)	1	81	(7.1–415.0)
Median viral load (log_{10} copies/mL)	3.2	0	0.02	4.5	0	< 0.01	6.4	0	< 0.01	6.5	0	< 0.01
Serum (n = 53)												
RT-qPCR (+) (n = 22)	12	10	0.4	13	9	< 0.01; 5	7	15	< 0.01; 1.5	8	14	< 0.01; 17.1
RT-qPCR (–) (n = 31)	13	18		7	24	(1.5–16.4)	0	31	(1.1–2.0)	0	31	(2.0–151.0)
Median viral load (log_{10} copies/mL)	0	0	0.5	2.3	0	< 0.01	2.6	0	< 0.01	2.6	0	< 0.01
Stool (n = 94)												
RT-qPCR (+) (n = 82)	46	36	< 0.01; 14.1	39	43	0.5	16	66	1	17	65	0.5
RT-qPCR (–) (n = 12)	1	11	(1.7–114.0)	4	8		2	10		1	11	
Median viral load (log_{10} copies/mL)	7.5	5	< 0.01	7.2	5	0.1	8	7	0.05	8.3	6.9	< 0.01
Urine (n = 111)												
RT-qPCR (+) (n = 32)	23	9	< 0.01; 3.8	15	17	0.8	7	25	0.4	7	25	0.4
RT-qPCR (–) (n = 79)	32	47	(1.5–9.2)	34	45		12	67		11	68	
Median viral load (log_{10} copies/mL)	0	0	< 0.01	0	0	0.7	0	0	0.3	0	0	0.4

[a]RT-qPCR, quantitative reverse transcription–polymerase chain reaction; SARS, severe acute respiratory syndrome; Y, yes; N, no; OR, odds ratio; CI, 95% confidence interval; NPA, nasopharyngeal aspirates

Table 4. Correlation of RT-qPCR clinical specimens taken during days 10 to 15 and laboratory values in 154 patients with SARS[a]

Specimen	Lymphopenia			Hepatic dysfunction			Abnormal urinalysis results		
	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)
NPA (n = 142)									
RT-qPCR (+) (n = 60)	56	4	0.8	40	20	< 0.01; 2.5 (1.2-5.2)	43	17	0.07
RT-qPCR (–) (n = 82)	70	12		38	44		52	30	
Median viral load ($\log_{10}$ copies/mL)	0	0	0.3	2.7	0	< 0.01	0	0	0.1
Serum (n = 53)									
RT-qPCR (+) (n = 22)	20	2	0.7	12	10	1	18	4	1
RT-qPCR (–) (n = 31)	26	5		19	12		26	5	
Median viral load ($\log_{10}$ copies/mL)	0	0	0.4	0	0	0.9	0	0	0.5
Stool (n = 94)									
RT-qPCR (+) (n = 82)	75	7	0.2	46	36	0.1	62	20	0.2
RT-qPCR (–) (n = 12)	9	3		4	8		7	5	
Median viral load ($\log_{10}$ copies/mL)	7.2	8.2	0.5	7.7	6.6	< 0.01	7.6	3.1	< 0.01
Urine (n = 111)									
RT-qPCR (+) (n = 32)	29	3	0.7	20	12	0.2	29	3	< 0.01; 7.2 (1.6–32.9)
RT-qPCR (–) (n = 79)	69	10		38	41		50	29	
Median viral load ($\log_{10}$ copies/mL)	0	0	0.3	0	0	0.2	0	0	< 0.01

[a]RT-qPCR, quantitative reverse transcription–polymerase chain reaction; SARS, severe acute respiratory syndrome; Y, yes; N, no; OR, odds ratio; CI, 95% confidence interval; NPA, nasopharyngeal aspirates.

part of an inflammatory "spillover" from virus-induced immune dysfunction or excessive cytokine activation in the lungs (18). However, our findings suggest that viral replication in extrapulmonary sites may be important, since viral load in stool is highly correlated with diarrhea, and electron microscopy of the ileum and colonic biopsy specimens from SARS patients showed numerous intracellular and extracellular virus particles (19). High viral load in urine is also associated with abnormal urinalysis findings. In this regard, SARS-CoV is currently known to grow only in fetal monkey kidney cells (i.e., fRhK4 or Vero E6) and the colonic carcinoma cell line (CACO-2). The correlation of viral load in stool with hepatic dysfunction is not completely unexpected, since high viral load in the stool is likely to be associated with significant portal venous viremia. In fact, viral load in stool is also associated with death.

Serum viral load correlates with oxygen desaturation, mechanical ventilation, and death. This finding is not surprising, since viremia has also been reported in adenovirus, respiratory syncytial virus (RSV), and rotavirus infections (20–22). However, viremia is reported to be short-lasting in mucosal infections. In one study, 5 out of 41 neonates with positive RSV antigen in nasal wash specimens were positive for RSV RNA in blood (21). High levels of adenovirus DNA in serum was also associated with death in children in whom adenovirus infection developed after allogeneic stem-cell transplantation. Six (86%) of 7 children who died of adenovirus infection, compared with 2 (7%) of 29 other patients, had high serum levels of adenoviral DNA ($p < 0.0001$) (20). The lack of association between viral load and lymphopenia at day 10 can be explained by the routine use of steroids, which induce apoptosis of lymphocytes. The apparently inferior performance of serum viral load as a prognostic indicator could be related to fewer available serum samples in this cohort. However, 38% of this group of 53 patients had oxygen desaturation; this proportion is not significantly different from the 142 patients (46%) who had submitted nasopharyngeal samples from day 10 to day 15.

Compared with other common viral respiratory diseases, the onset of peak viral load in the nasopharynx of SARS patients appears to be delayed. In a prospective study of viral shedding in nasopharyngeal secretions in experimental adult RSV infections, as measured by 50% tissue culture infective dose ($TCID_{50}$) viral titer or RT-qPCR, RSV is detected from day 2 to day 12, with a plateau phase from day 3 to day 8, at a peak viral load of 5 $\log_{10}$ copies/mL (23). In the case of experimental adult influenza, viral replication in NPA peaked ≈48 hours after the onset of symptoms and declined sharply thereafter, with an insignificant amount of viral shedding after day 8. Peak virus titers in symptomatic volunteers infected with influenza A H3N2 were $10^{2.5}$–$10^{7.0}$ $TCID_{50}$/mL of nasopharyngeal wash. Viral load was positively correlated with symptoms of fever and malaise as well as the amount of viral shedding (24). However, the reported low incidence of viremia and the early peak nasopharyngeal viral load in

Table 5. Correlation of RT-qPCR clinical specimens taken during days 10 to 15, and clinical manifestations in 40 patients with SARS who had specimens taken from all four anatomical sites[a]

	Diarrhea			O_2 desaturation			Mechanical ventilation			Death		
Specimen	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)	Y	N	p value; OR (CI)
NPA (n=40)												
RT-qPCR (+) (n = 18)	11	7	0.4	11	7	0.02; 5.3 (1.4–21.1)	7	11	< 0.01; 1.6 (1.1–2.4)	8	10	< 0.01; 1.8 (1.2–2.7)
RT-qPCR (–) (n = 22)	10	12		5	17		0	22		0	22	
Median viral load (log_{10} copies/mL)	2.9	0	0.4	5.1	0	< 0.01	6.4	0	< 0.01	6.4	0	< 0.01
Serum (n = 40)												
RT-qPCR (+) (n = 18)	12	6	0.1	11	7	0.02; 5.3 (1.4–21.1)	7	11	< 0.01; 1.6 (1.1–2.4)	8	10	< 0.01; 1.8 (1.2–2.7)
RT-qPCR (–) (n = 22)	9	13		5	17		0	22		0	22	
Median viral load (log_{10} copies/mL)	2.3	0	0.3	2.4	0	0.02	2.6	0	< 0.01	2.6	0	< 0.01
Stool (n = 40)												
RT-qPCR (+) (n = 30)	20	10	< 0.01; 18 (2.0–16.3)	12	18	1	5	25	1	7	23	0.7
RT-qPCR (–) (n = 10)	1	9		4	6		2	8		1	9	
Median viral load (log_{10} copies/mL)	7.8	2.6	< 0.01	7.3	7.2	0.5	8.2	7.2	0.5	8.7	7	0.03
Urine (n = 40)												
RT-qPCR (+) (n = 9)	6	3	0.5	3	6	0.7	1	8	1	2	7	1
RT-qPCR (–) (n = 31)	15	16		13	18		6	25		6	25	
Median viral load (log_{10} copies/mL)	0	0	0.4	0	0	0.6	0	0	0.5	0	0	1

[a]RT-qPCR, quantitative reverse transcription–polymerase chain reaction; SARS, severe acute respiratory syndrome; Y, yes; N, no; OR, odds ratio; CI, 95% confidence interval; NPA, nasopharyngeal aspirates.

these two conditions could be explained by inherent characteristics of viral replication, background IgG and IgA against cross-reactive homologous antigens from previous infections, or innate immunity of the host. In many of these experimental infections in which the profile of viral load in NPA was documented, volunteers were adults who had low-level background antibodies and concomitant cell-mediated immunity against influenza or RSV (23,24).

Table 6. Correlation of RT-qPCR clinical specimens taken during days 10 to 15 and laboratory values in 40 patients with SARS who had specimens taken from all four anatomic sites[a]

	Lymphopenia			Hepatic dysfunction			Abnormal urinalysis results		
Specimen	Y	N	p value	Y	N	p value	Y	N	p value
NPA (n=40)									
RT-qPCR (+) (n = 18)	15	3	0.6	10	8	0.8	16	2	0.4
RT-qPCR (–) (n = 22)	20	2		11	11		16	6	
Median viral load (log_{10} copies/mL)	2.9	0	0.9	0	0	0.9	0.8	0	0.5
Serum (n = 40)									
RT-qPCR (+) (n = 18)	16	2	1	10	8	0.8	16	2	0.4
RT-qPCR (–) (n = 22)	19	3		11	11		16	6	
Median viral load (log_{10} copies/mL)	0	0	0.6	0	0	0.7	1.1	0	0.3
Stool (n = 40)									
RT-qPCR (+) (n = 30)	27	3	0.6	18	12	0.3	25	5	0.2
RT-qPCR (–) (n = 10)	8	2		3	7		6	4	
Median viral load (log_{10} copies/mL)	7.9	7.2	0.8	7.8	4.7	0.04	7.8	2.6	0.1
Urine (n = 40)									
RT-qPCR (+) (n = 9)	8	1	1	7	2	0.1	8	1	0.7
RT-qPCR (–) (n = 31)	27	4		14	17		23	8	
Median viral load (log_{10} copies/mL)	0	0	1	0	0	0.06	0	0	0.4

[a]RT-qPCR, quantitative reverse transcription–polymerase chain reaction; SARS, severe acute respiratory syndrome; Y, yes; N, no; NPA, nasopharyngeal aspirates.

Falsely negative serum viral load in influenza or RSV infection could be related to hemagglutinating properties of these two viruses, which may remain stuck on the erythrocyte in the clotted blood sample. In a novel emerging infectious disease such as SARS, most of the general population would not have background or partial immunity (2,25); viremia or a delayed peak viral load at day 10 in NPA is therefore not completely unexpected.

One limitation of the present study is its retrospective nature. Only specimens provided at approximately day 10 could be tested and analyzed. Changes of lymphocyte subsets were also not analyzed. Nonetheless, lymphocyte changes in SARS patients were well reported by two other groups who showed a consistent decrease in the peripheral blood level of dendritic cell subsets, natural killer cells, CD4+ and CD8+ T lymphocytes, and B lymphocytes (26,27).

SARS is predominantly a respiratory infection, which possibly spreads through the mucosal lumen or the bloodstream to extrapulmonary sites where viral replication leads to nonrespiratory manifestations. Concomitant immune dysregulation and associated inflammatory damage could accentuate disease progression and death. High viral load in NPA, with or without high viral load in serum, is a useful prognostic indicator of respiratory failure or death. The presence of viral RNA in multiple body sites also indicates poor prognosis. Early treatment with an effective antiviral agent before day 10 may decrease the peak viral load, ameliorate symptoms, and improve outcome; early treatment may also reduce viral shedding and thus the risk for transmission.

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Severe Acute Respiratory Syndrome

SARS Antibody Test for Serosurveillance

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A peptide-based enzyme-linked immunosorbent assay (ELISA) can be used for retrospective serosurveillance of severe acute respiratory syndrome (SARS) by helping identify undetected chains of disease transmission. The assay was developed by epitope mapping, using synthetic peptides from the spike, membrane, and nucleocapsid protein sequences of SARS-associated coronavirus. The new peptide ELISA consistently detected seroconversion by week 2 of onset of fever, and seropositivity remained through day 100. Specificity was 100% on normal blood donor samples, on serum samples associated with infection by other pathogens, and on an interference panel. The peptide-based test has advantages of safety, standardization, and automation over previous immunoassays for SARS. The assay was used for a retrospective survey of healthy healthcare workers in Taiwan who treated SARS patients. Asymptomatic seroconversions were detected in two hospitals that had nosocomial disease.

Retrospective surveillance for infection is an important means to screen for and interrupt undetected chains of disease transmission. Such surveillance may be key to tracking the severe acute respiratory syndrome–associated coronavirus (SARS-CoV) because mild and asymptomatic cases of SARS-CoV infection that do not meet the World Health Organization's case definition (1) have been identified by immunoassays (2–4), and SARS-CoV–like viruses have been isolated from wild mammals (5). SARS-CoV may have persisted over the summer in previously affected areas in such difficult-to-recognize reservoirs (6). The reemergence of SARS in the city of Guangzhou of the Guangdong Province of China in December 2003 and January 2004 (7) is evidence that an unknown reservoir exists and signals the need for continued surveillance with laboratory testing.

The current laboratory methods for identifying SARS are not ideal tools for use in retrospective mass screening. Reverse transcription–polymerase chain reaction (RT-PCR) for detecting viral RNA is the most sensitive method for early identification of SARS. However, viral load rapidly declines beginning 9 or 10 days after disease onset (8–10). Moreover, RT-PCR requires sophisticated equipment and high laboratory quality-assurance standards (11,12). Identifying seroconversion to SARS-CoV by immunoassay also is a definitive criterion for laboratory determination of SARS (13), and seroconversion is the preferred standard for retrospectively detecting SARS-CoV infection (14). SARS immunoassays include the enzyme-linked immunosorbent assay (ELISA) or Western blot with antigen from whole virus or various recombinant proteins, a cumbersome immunofluorescence assay (IFA) using whole virus fixed on glass, and methods to determine neutralizing antibodies (10,11). Immunoglobulin (Ig) G to SARS-CoV, detected by these immunoassays, begins to rise sharply by day 11 after onset of symptoms. Virtually all SARS patients show virus-specific antibody by week 3, and anti–SARS-CoV IgG persists through day 100 (8,10,15). Although any of these immunoassays can provide a definitive laboratory finding, all but the recombinant tests require biosafety level 3 to contain the virus or are time-consuming to perform, have not been well-standardized, are of unknown specificities, and would be difficult to adapt to large-scale manufacture. Improving laboratory methods for the large-scale serologic surveillance of SARS, particularly in the presence of other respiratory illnesses, and standardization of diagnostic assays are key priorities for controlling SARS (16). In this report,

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a standardized and rapid peptide-based SARS ELISA is characterized for sensitivity and specificity.

Beginning in April 2003, delay in recognizing SARS cases and in implementing isolation procedures led to several nosocomial clusters of SARS-CoV transmission in healthcare facilities in Taiwan (17,18). The results from a retrospective serologic survey by the peptide ELISA of healthcare workers from facilities affected by nosocomial outbreaks are presented as a working example.

Materials and Methods

Synthetic Peptide ELISA

We developed an ELISA for SARS that has synthetic peptide antigens as the solid-phase immunosorbent. Over 200 overlapping peptides, deduced from the Tor2 SARS-CoV genomic sequence (19), were synthesized as candidate antigens from the spike (S), membrane (M), and nucleocapsid (N) proteins. Candidate immunodominant S, M, and N peptides were selected and refined on the basis of serologic reactivities to a panel of serum samples from 13 patients clinically diagnosed with SARS at National Taiwan University Hospital in Taipei and the Xiaotangshan SARS Emergency Hospital in Beijing (20). Epitope mapping by serologic validation has been described for the development of peptide-based ELISA tests for HIV, hepatitis C virus, and foot-and-mouth disease virus (21–23). For the peptide-based SARS ELISA, wells of microtiter plates were coated with 2 µg/mL of a mixture of the S, M, and N protein–derived peptides, and serologic reactivities were determined by a standard ELISA procedure as previously described (23), except that the detector was horseradish peroxidase–conjugated goat anti-human IgG, and the chromaphore was 3,3′,5,5′-tetramethylbenzidine (TMB). In brief, serum samples, including two normal human samples provided as nonreactive controls, were diluted 1:20 in phosphate-buffered saline with carrier proteins and preservative. The diluted serum samples were reacted to the peptide-coated microtiter wells for 1 h at 37°C. Plates were washed 6 times, reacted to the antibody conjugate, again washed 6 times, and reacted to TMB; reactivity was then determined by A_{450}. Assay results were obtained within 3 h. Results were scored on the basis of the signal/cutoff (S/C) ratio, and cutoff absorbance was determined from the mean of the two controls plus 6 standard deviations (SD) from the distribution of normal human samples (Figure 1).

Serum Panels

Seroconversion panels were collected as serial serum samples from SARS patients at National Taiwan University Hospital in the course of treatment. These panels tested positive for seroconversion by IFA (24) and are used here to evaluate analytical sensitivity.

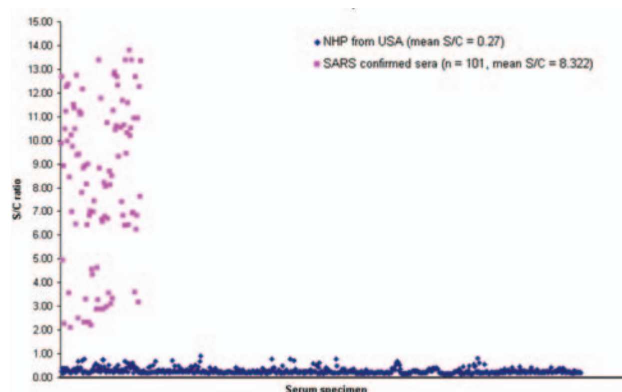

Figure 1. Distribution of tested specimens. Signal/cutoff (S/C) distribution of Center for Disease Control (Taiwan) SARS (severe acute respiratory syndrome) serum panel and blood donor serum panel. The mean signal/cutoff (S/C) ratio for the SARS samples was 8.08. The mean S/C for the 1,390 normal human plasma was 0.28.

A panel of 69 serum samples from convalescent SARS patients were provided by the Center for Disease Control, Department of Health, Taiwan, to evaluate diagnostic sensitivity. These serum specimens were confirmed for SARS-CoV infection clinically by the World Health Organization diagnostic criteria (1) and serologically by whole virus–based ELISA and IFA; some specimens were also confirmed by RT-PCR (24). Samples were drawn with appropriate timing for serologic reactivity (7–25 weeks after onset of symptoms). A panel of 1,390 plasma samples collected from random blood donors in Florida before 2001 was obtained from the Gulf Coast Regional Blood Center (Houston, TX) for specificity evaluation.

Additional specificity studies were conducted with serum that had serologic reactivities for bloodborne pathogens (HIV-1, HIV-2, hepatitis C virus, HTLV 1/II, and syphilis) obtained by United Biomedical Inc. before 2000 from various U.S. sources, an interference panel supplied by Boston Biomedica Inc. (Boston, MA) of serum samples with interference substances commonly found in processed clinical samples (EDTA, acid citrate dextrose, and citrate phosphate dextrose with adenine), and serum supplied by National Taiwan University Hospital from patients associated with typical and atypical respiratory pathogens other than SARS-CoV (influenza, rubella, cytomegalovirus, Epstein-Barr virus, and *Mycoplasma pneumoniae*). Serum samples were collected from healthy healthcare workers after interviews to confirm lack of signs and symptoms of SARS including fever, respiratory symptoms, and diarrhea.

Results

Sensitivity

The peptide-based ELISA was evaluated for sensitivity to seroconversion on eight seroconversion panels obtained from National Taiwan University Hospital (Figure 2, Table 1). In patient 1, seroconversion was detected by day 11 with an A_{450} of 1.638. Absorbance remained at >2 at day 97. In patient 2, from whom blood was drawn on days 0, 6, 16, 27, and 116 (no samples were collected from day 6 to day 16), seroconversion was apparent on day 16 after the onset of fever. On day 116, the A_{450} remained >3. In five of the other six seroconversion panels, from acute to convalescent phases, seropositivity was observed by days 8 to 12, and by day 16 in patient 6, from whom serum had not been collected from days 6 to 16 (Figure 2). The peptide-based ELISA showed an analytical sensitivity to earliest time of detection by week 2 and for duration of detection beyond day 100. The seroreactivities of patients 3 to 8 were also evaluated by a standard IFA method (24) for comparison. Seroconversion was detected in all six patients by the IFA method within 2 days of the peptide ELISA (data not shown).

The diagnostic sensitivity of the peptide ELISA was 100% on a panel of 69 convalescent-phase serum samples from SARS patients provided as a reference panel by the Center for Disease Control, Department of Health, Taiwan. These sera, confirmed for SARS by diagnostic and serologic criteria, displayed a mean S/C ratio of 8.08 (Figure 1).

Table 1. Sensitivity of peptide-based SARS-CoV ELISA from two patients at NTU[a]

Patient/day	A_{450nm}	S/C ratio
SARS patient 1		
Day 0	0.119	0.44
Day 11	1.638	6.09
Day 17	2.447	9.10
Day 38	2.749	10.22
Day 97	2.600	9.67
SARS patient 2		
Day 0	0.068	0.25
Day 6	0.163	0.61
Day 16	0.345	1.28
Day 27	1.212	4.51
Day 116	>3.000	>9.40

[a]SARS, severe acute respiratory syndrome; CoV, coronavirus; ELISA, enzyme-linked immunosorbent assay; S/C = signal/cutoff ratio; NTU, National Taiwan University.

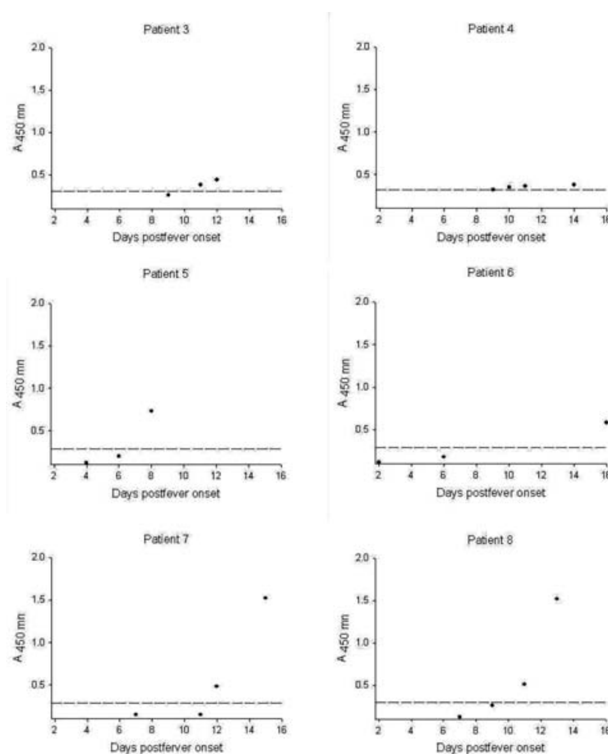

Figure 2. Time of seroconversion from onset of fever for patients infected with severe acute respiratory syndrome–associated coronoavirus. Cutoff absorbance shown by dotted line.

Specificity

The specificity of the peptide-based SARS ELISA was tested on plasma obtained before 2001 from 1,390 random Florida blood donors (Gulf Coast Regional Blood Center, Houston, TX). These normal plasma samples with a presumed zero seroprevalence rate gave a mean A_{450} of 0.074 ± 0.0342 (SD). Subsequently, the cutoff value for the peptide-based assay was set as the mean A_{450} for duplicate nonreactive controls plus 0.279 (based on 6 SDs from the mean for these 1,390 normal plasma samples). The distribution of the S/C ratio for the blood bank samples is plotted in Figure 1 with the mean S/C ratio of 0.28. None showed positive reactivity, for a specificity on the normal samples of 100% (Table 2).

The peptide ELISA was further evaluated for specificity with a pre-2000 collection of serum samples from patients with seropositivities for bloodborne pathogens such as HIV-1, HIV 2, hepatitis C virus, HTLV 1/II, and syphilis (various U.S. sources), and with normal serum samples spiked with interference substances heparin, EDTA, ACD, and CPDA-1 (Boston Biomedica). The 52 samples with seroreactivities for the pathogens all tested negative by the peptide-based SARS ELISA, as did the 41-sera interference panel (Table 2).

The peptide ELISA was evaluated for specificity on serum samples drawn from patients associated with typical and atypical respiratory pathogens other than SARS-CoV (National Taiwan University Hospital). These included samples from 1) 10 patients naturally infected with influenza (two sequential bleeds per influenza patient), 2) 10 patients with rubella, 3) 8 patients with cyto-megalovirus infection, 4) 9 patients with Epstein-Barr virus, 5) 5 patients infected with *Mycoplasma pneumoniae*, a

Table 2. Sensitivity and specificity of peptide-based ELISA[a]

Source of samples	Total no.	ELISA+	ELISA–
Blood donors (Gulf Coast Regional Blood Bank, USA)	1,390	0	1,390
Blood-transmitted pathogen panel (various blood banks, USA)	52	0	52
Interference panel (BBI)	41	0	41
Confirmed SARS (TW CDC)	69	69	0
Influenza patients (NTU)	10	0	10
Influenza vaccinees (NTU)	16	0	16
Rubella patients (NTU)	10	0	10
EBV patients (NTU)	9	0	9
Mycoplasma (NTU)	5	0	5
CMV patients (NTU)	8	0	8

[a]ELISA, enzyme-linked immunosorbent assay; NTU, National Taiwan University; BBI, Boston Biomedica Inc (Boston, MA); TW CDC, Taiwan Center for Disease Control; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

bacterial agent for atypical pneumonia, and 6) pre- and postvaccine blood samples from 16 patients given influenza vaccine. All samples were tested in duplicate. The site-specific antigens of the peptide SARS-CoV ELISA were free of cross-reactivities to the other respiratory pathogens (Table 2).

Serologic Survey of Healthcare Workers

A prospective study was performed to determine asymptomatic infection among primary healthcare workers in hospitals that treated SARS patients. We collected serum samples from 623 healthcare workers without symptoms, not all of whom were in direct contact with SARS patients, who agreed to be tested for antibody to SARS-CoV at Ho Ping, Yang Ming, En Chu Kong, and Hsin Tai Hospitals, approximately 4 weeks after the outbreaks were recognized. Ho Ping and Yang Ming Hospitals had admitted SARS patients before the recognition of SARS and before healthcare workers had implemented control measures. Subsequently, these facilities experienced transmission to healthcare workers. The En Chu Kong and Hsin Tai facilities admitted patients once control measures had been implemented. Neither of these hospitals recorded transmission of SARS to healthcare workers.

ELISA detected three cases out of 383 samples from Ho Ping and one case in 50 blood samples from nursing aides at Yang Ming. These four positive samples, indicative of asymptomatic infection, were confirmed for seropositivity by IFA. None of the 190 serum samples from the two hospitals without nosocomial infection displayed seroconversion.

Discussion

A convenient ELISA to detect IgG to SARS-CoV, based on site-specific synthetic antigens taken from the S, M, and N proteins of the virus, has high specificity. No cross-reactivity was detected in samples associated with common non-coronavirus respiratory pathogens. In addition, the lack of detectable reactivities among the 1,390 U.S. blood donors supports a specificity for the assay to distinguish SARS-CoV infection from infection by other human coronaviruses. The presence of anti-coronavirus antibodies among a U.S. population of this size is strongly anticipated because an incidence as high as 8% for OC43 and 229E respiratory infections has been observed, even among healthy young adults (25).

The new peptide-based ELISA is equivalent in sensitivity to other immunoassays for SARS and can be detected after day 100. The synthetic antigens provide the advantages of high standardization, freedom from biohazard, and ease of scale-up production. Moreover, testing by the ELISA format can be readily automated for large-scale screening. The highly specific peptide-based SARS antibody test is a convenient means to carry out widespread retrospective surveillance, such as that now being proposed for China to trace hotspots of persons carrying antibodies to SARS-CoV and to track the origins of the disease (26).

A preliminary survey with the peptide ELISA detected asymptomatic clusters of seroconversion among exposed healthcare workers in two Taiwan hospitals that also had nosocomial disease. In contrast, no seroconversion was found among the exposed healthcare workers from two hospitals that had no apparent disease transmission to healthcare workers. The finding of asymptomatic seropositive persons indicates that the test will be useful in larger retrospective surveillance studies, which are needed to fully define the epidemiology and spectrum of disease.

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The protocol and the informed consent documents were approved by the Ethics Committee of Medical Research of the Biomedical Sciences, Academia Sinica. All participation was voluntary and was documented with written informed consent.

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Experimental Infection of Ground Squirrels (*Spermophilus tridecemlineatus*) with Monkeypox Virus

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A proposed new small-animal (rodent) model for studying the pathogenesis and treatment of severe orthopoxvirus infections is described. Thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were infected intraperitoneally and intranasally with monkeypox virus (MPXV). A fulminant illness developed in all animals, and they died 6–9 days after infection. Virus was cultured from the blood and oropharynx several days before death; at necropsy, all of the organs tested contained relatively high titers of MPXV. The major pathologic findings were in the liver, which showed centrilobular necrosis, steatosis, and basophilic inclusion bodies in hepatocytes. Splenic necrosis was also observed, as well as interstitial inflammation in the lungs. The pathologic features of MPXV in ground squirrels are similar to that described with MPXV in macaques and severe variola (smallpox) virus infection in humans.

Until last year, human monkeypox was confined to forested areas of central and West Africa, where sporadic epizootics have occurred (1). However, in 2003 monkeypox appeared in the United States, and 32 human cases were confirmed during an outbreak that occurred in pet owners in the Midwest (2,3). Imported African rodents were implicated as the probable source of the outbreak, although the virus also infected other wild animal pets (i.e., prairie dogs) that had contact with them (4). On the basis of these reports and earlier studies in Africa (5–7) that suggest that squirrels and certain other wild rodents might be reservoirs of monkeypox virus (MPXV), we tested the susceptibility of several North American wild rodent species to MPXV infection. We report the results of our studies with the common thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*.

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Materials and Methods

Animals

Ten adult thirteen-lined ground squirrels (*S. tridecemlineatus*) were used in the experiment. The animals were wild-caught and purchased from a commercial supplier (TLS Research, Bloomington, IL). Ground squirrels were housed individually in filter-bonneted, solid bottom (123-cm^2 floor area) plastic cages in an isolation room within an animal biosafety level 3 facility. All persons handling the animals had recently received smallpox (vaccinia) vaccination and used appropriate personal protection. Animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

Virus

The strain of MPXV used was designated MPX 2003 and was provided by the Centers for Disease Control and Prevention, Atlanta, Georgia. This virus was originally isolated from a skin lesion from a human monkeypox patient during the 2003 U.S. outbreak (3). A stock of the virus was prepared from infected Vero cells; the unsonicated frozen cell lysate was used to infect the rodents and had a titer of $10^{6.1}$ PFU/mL.

Virus Assay

Samples for virus assay were stored at –80°C. Before testing, tissue samples were thawed and triturated in sterile Ten Broeck glass tissue grinders in phosphate-buffered saline (PBS), pH 7.4, containing 30% heat-inactivated

(56°C for 30 min) fetal bovine serum (FBS) to prepare an approximate 10% (wt/vol) tissue homogenate. After centrifugation at 6,000 rpm for 5 min to clarify the suspension, serial 10-fold dilutions from 10^{-1} to 10^{-8} were prepared in PBS containing 10% FBS. Similar dilutions were made with the blood and throat swab suspensions for virus assay.

Dilutions of the tissue homogenates, blood, and throat swab suspensions were titrated in 24-well cultures of Vero cells; four wells were used for each dilution, as described (8). Cultures were incubated at 37°C, and plaques were counted 4–6 days later. Virus titers were defined as the number of PFU per milliliter of sample.

Experimental Infection of Animals

Ground squirrels were infected by the intraperitoneal (IP) or intranasal (IN) routes. Five squirrels were injected IP with $10^{5.1}$ PFU of MPX 2003 virus. Five other animals were infected by the IN route; under Halothane (Halocarbon Laboratories, River Edge, NJ) anesthesia, two drops of the stock virus solution containing $10^{6.1}$ PFU/mL were instilled into each nostril. After infection, all rodents were observed daily for signs of illness; if an animal died, a necropsy was performed, and tissues (liver, spleen, kidney, adrenal, lung, heart, and brain) were taken for histopathologic examinations and virus titration. In some animals, enlarged mesenteric lymph nodes and thymus were also taken. Blood (100 µL from the retroorbital sinus) and an oropharyngeal swab were also taken daily from each animal for virus assay. The whole blood and the swab were expressed in 900 µL of PBS with 10% FBS.

Histopathologic and Immunohistochemical Methods

At necropsy, tissue samples were taken from the animals and preserved in 10% buffered formalin for 24 to 48 h, followed by storage in 70% ethanol. After fixation, the samples were processed for routine embedding in paraffin. Four- to 5-µm–thick tissue sections were made and stained by the hematoxylin and eosin method (9).

Selected tissue sections were also studied immunohistochemically, by using a vaccinia hyperimmune mouse ascetic fluid, at a dilution of 1:100. A mouse-on-mouse IHC-ISO labeling kit (InnoGenex, San Ramon, CA) was used, according to the manufacturer's instructions and a protocol similar to one described (9). The primary antibody (a mouse antivaccinia ascitic fluid) was incubated with the section at 4°C for overnight. Tissue sections from two uninfected animals were used as negative controls.

Transmission Electron Microscopy (EM)

For EM, formalin-fixed tissues were additionally fixed in a mixture of 2.5% formaldehyde, 0.1% glutaraldehyde, 0.03% trinitrophenol, and 0.03% $CaCl_2$ in 0.05 mol/L cacodylate buffer, postfixed in 1% OsO_4 in 0.1 mol/L cacodylate buffer, stained en bloc in 1% uranyl acetate in 0.1 mol/L maleate buffer, dehydrated in ethanol, and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert-Leica Ultracut S ultramicrotome and examined in a Philips 201 electron microscope at 60 kV.

Results

Clinical Manifestations

Most of the animals became lethargic and anorexic within 4 or 5 days of infection; however, detectable skin lesions, respiratory distress, or other obvious symptoms of disease did not develop in any of the ground squirrels. The five animals (numbers 11–15) infected IP were moribund or dead within 6 or 7 days after infection; death occurred in the IN-infected group (animals 16–20) approximately 2 days later (Table 1). All of the animals were dead within 9 days of infection.

Virus Titrations

Table 1 shows the amount of virus detected in daily blood and throat swab samples taken from the infected rodents. Among the IP-infected squirrels, virus was first detected in the blood on day 3; in contrast, in the IN-infected animals, virus was first detected in the oropharynx (throat swab) on days 2 to 4. Virus titers in the two groups of animals were similar and tended to increase with time.

Table 2 gives the results of virus titrations performed on 10% suspensions of liver, spleen, kidney, lung, heart, and brain taken at necropsy from infected animals. The highest MPXV titers were found in the liver and spleen, but relatively large amounts of virus were detected in the other organs as well. The amount of virus present in the various organ groups (i.e., liver or lung) did not appear to be related to the route of infection, a finding that suggests that the MPXV infection in the rodents was disseminated. The lower virus titers in brain and heart, compared to blood, suggest that MPXV did not replicate appreciably in those organs.

Pathologic Changes and Immunohistochemical Analysis

In the IP-infected animals (numbers 11–15), considerable centrilobular hepatocytic degeneration or necrosis occurred in the liver. Many hepatocytes, particularly in the areas of degeneration, contained round to oval-shaped basophilic inclusion bodies of various sizes in the cytoplasm. Inflammatory cell infiltration in the lobules was minimal. The portal tracts were normal. Moderate-to-marked necrosis of the spleen was also present in all the animals (Figure 1B). This necrosis was characterized by lymphocytic karyorrhexis in the white pulp, and fibrinoid

Table 1. Results of virus titrations performed on blood (B) and throat swab (TS) samples from monkeypox virus–infected ground squirrels[a]

Animal no.[b]	Sample	Day after infection								
		1	2	3	4	5	6	7	8	9
11	B	0.7[c]	0	1.4	2.9	4.0	D			
	TS	NT	0	0	3.1	5.3				
12	B	0	0	1.0	2.9	3.8	4.8	D		
	TS	NT	0	0	2.2	3.9	5.8			
13	B	0	0	1.2	3.2	4.2	D			
	TS	NT	0	0	3.9	5.8				
14	B	0	0	0.7	2.5	3.9	D			
	TS	NT	0	0	2.9	5.2				
15	B	0	0	0.7	3.0	3.9	D			
	TS	NT	0	0	2.5	6.0				
16	B	0	0	0	0	1.6	2.1	4.2	D	
	TS	NT	0	0	1.7	3.7	4.6	5.3		
17	B	0	0	0	0.7	2.1	2.7	5.0	NT	D
	TS	NT	0	1.7	2.2	4.0	4.9	5.7	6.2	
18	B	0	0	0	0	2.0	2.5	4.3	D	
	TS	NT	0	1.0	1.9	2.9	3.5	4.6		
19	B	0	0	0	0	0	1.9	4.7	4.2	D
	TS	NT	1.8	2.3	3.0	3.9	4.0	5.4	4.9	
20	B	0	0	0	0	1.0	2.5	5.1	4.8	D
	TS	NT	2.5	1.7	2.5	3.7	3.9	3.7	5.4	

[a]NT, not tested; D, animal dead.
[b]Animals 11–15 were infected intraperitoneally; animals 16–20 were infected intranasally.
[c]Virus titer expressed as $\log_{10}$ PFU/mL of sample. Values $<10^{0.7}$ were beyond the sensitivity of the assay and were marked as zero.

necrosis, congestion, and endothelial cell swelling in the red pulp, accompanied by cell debris. The lungs showed mild-to-multifocal thickening of the alveolar septa and focal consolidations.

In contrast, the livers of the IN-infected animals (numbers 16–20) exhibited multifocal steatosis; some had a periportal distribution, while others were mainly microvesicular in pattern. In addition, four of the five animals exhibited diffuse hepatocytic necrosis; only one liver (animal 19) had centrilobular necrosis. The characteristic cytoplasmic inclusion bodies were present in all livers (Figure 1A). As observed in the IP-infected animals, moderate-to-severe splenic necrosis occurred. In addition to the variable consolidation and interstitial inflammation in the lungs, some of the IN-infected animals showed necrosis in the peribronchial lymphoid tissue. Lymph nodes from other sites (i.e., mediastinal brown fat) also showed focal necrosis, accompanied by proliferation of immunoblast-like cells, fibroblasts, and macrophages.

Immunohistochemically, no positive staining was observed in control animals. Sections of adrenals, kidneys, and hearts from the infected ground squirrels were also negative. In the liver, most of the larger inclusion bodies stained strongly positive for viral antigen (Figure 1C); however, some of the smaller inclusion bodies were negative. Depending on the severity of the histologic abnormality, this positive staining sometimes involved the surrounding cytoplasm and cytoplasmic membranes. The spleen also stained strongly positive; the intensity generally corresponded to the severity of pathologic changes (Figure 1D). In some animals, the cells lining the surface of the splenic capsule (mesothelial cells) were enlarged

Table 2. Results of virus titrations performed on 10% organ suspensions of 10 monkeypox virus–infected ground squirrels[a]

Animal no.[b]	Liver	Spleen	Kidney	Lung	Heart	Brain
11	7.5[a]	6.8	4.4	5.9	3.5	2.4
12	7.2	6.4	4.9	6.1	3.8	1.7
13	7.9	6.7	5.3	5.9	4.5	1.7
14	7.5	6.8	5.0	6.1	3.6	2.6
15	7.9	6.8	4.7	6.7	4.2	4.0
16	7.6	6.4	4.7	6.1	4.2	2.5
17	7.4	6.4	4.8	5.5	3.9	2.0
18	7.8	6.4	5.4	6.0	4.2	2.8
19	7.0	6.4	4.1	5.6	4.1	2.1
20	7.8	6.8	4.9	6.1	5.7	1.7

[a]Samples taken at death (necropsy). Virus titer expressed as $\log_{10}$ PFU/mL of 10% tissue suspension.
[b]Animals 11–15 were infected intraperitoneally; animals 16–20 were infected intranasally.

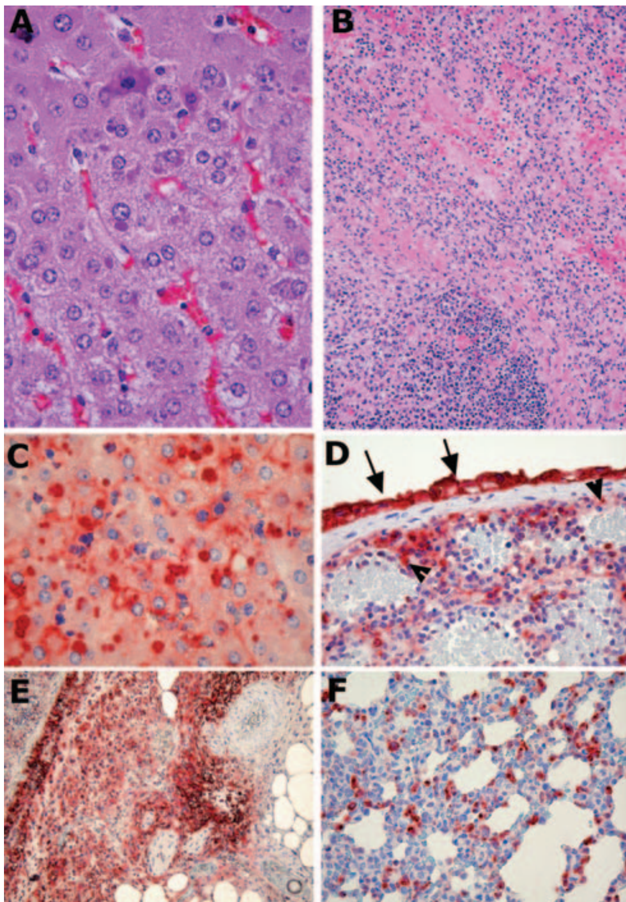

Figure 1. Representative photomicrographs of histologic changes and immunohistochemical staining of tissues from ground squirrels infected with monkeypox virus. A) Liver from a ground squirrel (intranasal infection) showing mild degenerative changes, including early steatosis, and purple-colored viral cytoplasmic inclusion bodies in the hepatocytes (40x objective). B) Spleen from a ground squirrel infected intraperitoneally, showing extensive necrosis (20x objective). C) Liver showing positive antigen staining of the intrahepatocytic inclusion bodies; antigen is present in the cytoplasm and to a lesser extent in cell membranes (40x objective). D) Spleen from a ground squirrel infected intraperitoneally, showing positive antigen staining in the interstitial cells, endothelial cells (arrowheads), and the surface mesothelial cells (arrows) (20x objective). E) Same tissue sample as D, showing the edge of the spleen with antigen-positive mesothelial layer; the adjacent fat and fibrous tissue show necrosis but are also strongly positive for antigen (20x objective). F) Lung from the same animal showing many antigen-positive interstitial cells and pneumocytes (40x objective). A and B, hematoxylin and eosin stain; C–F, immunoperoxidase stain.

and were also strongly positive for viral antigen. In these animals, the positive staining appeared to extend into the superficial zones of the neighboring tissues or organs, such as fat (Figure 1E), pancreas, and adrenal gland, which otherwise were generally negative for viral antigen and lacked pathologic changes. This finding suggests that virus spread directly between adjacent sites when the boundaries (capsules) were broken. Necrotic areas in the perisplenic and periadrenal fat also stained strongly positive.

Viral antigen staining in other organs was less consistent. In the lungs, scattered interstitial cells and a few alveolar pneumocytes were positive (Figure 1F). In the kidneys, sometimes rare mononuclear leukocytes in a few glomeruli were positive. However, these latter positive monocytes probably represented cells in circulation, rather than actual virus replication in the renal tissue.

Examination of selected tissues by EM confirmed the results of immunohistochemistry. In ultrathin sections, groups of poxlike virions were readily seen within cytoplasm of infected hepatocytes (Figures 2A and B).

Discussion

Results of our study indicate that the thirteen-lined ground squirrel is highly susceptible to MPXV. Experimental infection of the animals by both IP and IN routes produced a fulminant uniformly fatal disease. All of the animals were dead by the day 9. The amount and wide distribution of virus in various organs indicate that the infection was disseminated. Initially, the first recovery of MPXV from the blood of IP-infected squirrels and from the oropharynx of IN-infected animals suggested that the pathogenesis of MPXV might be different, depending on the route of infection; however, at necropsy, the amount of virus present in the respective organ systems of the two groups was similar. The histopathology observed at necropsy in the two groups was also similar, although squirrels in the IN-infected group had more hepatic steatosis and pulmonary consolidation. However, this difference may simply be a reflection of the longer incubation period and later death of the IN-infected animals.

In a recent publication, Guarner et al. (4) described the histopathologic findings in two sick prairie dogs (*Cynomys* spp.), collected from a pet store during the 2003 monkeypox outbreak in the United States. The abnormal pathologic findings in these two animals were ulcerative lesions on the tongue and conjunctiva and in the lung (bronchioalveolar pneumonia). MPXV was recovered from the lungs of both animals, but only mild inflammation in the liver and reactive hyperplasia in the spleen were found. In another study, we experimentally infected eight prairie dogs with MPXV by the IN and IP routes. Skin or mucosal lesions developed in some of these animals; some of them survived; in general, the survival time was longer and the degree of pathology was less than that observed in the ground squirrels (unpub. data). Although both rodent species are members of the family *Sciuridae,* these observations suggest that MPXV infection in the thirteen-lined ground squirrel is more severe than in prairie dogs.

The fulminant disease and pathology produced in *S. tridecemlineatus* by MPXV are similar to the pathologic

findings described in experimentally infected macaques (10), which in turn are similar to life-threatening or fatal smallpox (variola virus infection) in humans (11). This similarity suggests that ground squirrels might be an excellent small-animal model for studying the pathogenesis and treatment of severe orthopoxvirus infections in people. Concern about potential bioterrorism (12) as well as recent reports of zoonotic transmission of poxviruses (13) have renewed research interest in these viruses. *S. tridecemlineatus* is abundant in grassland and prairie habitats in the central United States and adjacent regions of Canada (14), so supply should be plentiful. Their adult weight (140–252 g), laboratory diet, and cage requirements are similar to those of a large hamster or small guinea pig. Thus, we feel that these animals have considerable value as a laboratory model.

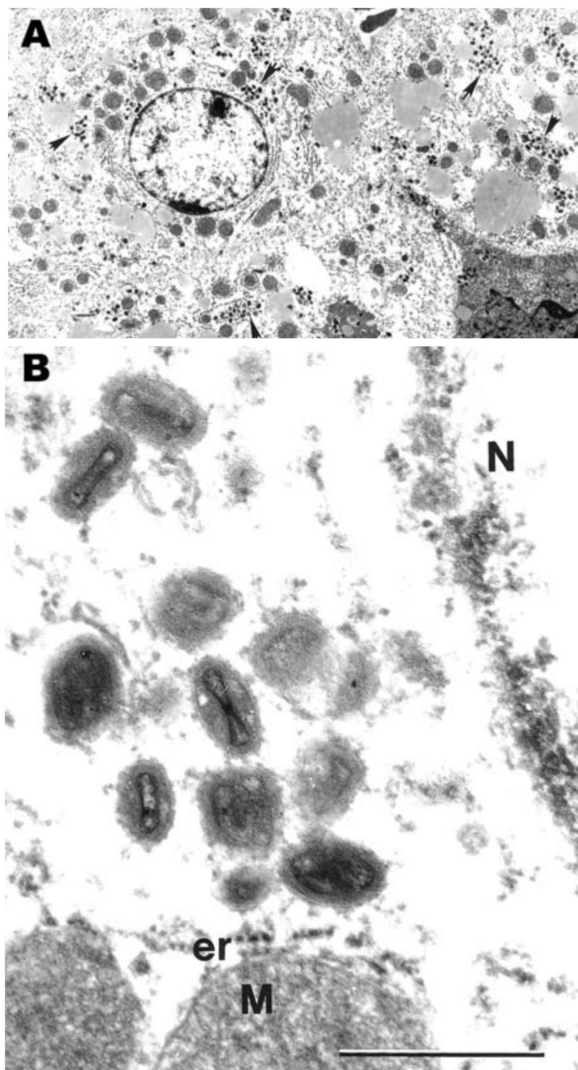

Figure 2. Ultrastructural localization of monkeypox virus in hepatocytes in the liver of a ground squirrel 5 days after infection. A) Hepatocytes contain numerous groups of virions (arrows) in their cytoplasm (bar = 1 μm). B) Magnified area of A, showing typical ultrastructure of monkeypox virus virions and characteristic hepatocyte mitochondria (M) surrounded by cisterns of granular endoplasmic reticulum (er). N, fragment of hepatocyte nucleus; bar = 0.5 μm.

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Dr. Tesh is professor of pathology at the University of Texas Medical Branch in Galveston. His research interests are the epidemiology and pathogenesis of arthropodborne and emerging zoonotic viral diseases.

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Silent Nucleotide Polymorphisms and a Phylogeny for *Mycobacterium tuberculosis*

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Much remains unknown of the phylogeny and evolution of *Mycobacterium tuberculosis*, an organism that kills 2 million people annually. Using a population-based approach that analyzes multiple loci around the chromosome, we demonstrate that neutral genetic variation in genes associated with antimicrobial drug resistance has sufficient variation to construct a robust phylogenetic tree for *M. tuberculosis*. The data describe a clonal population with a minimum of four distinct *M. tuberculosis* lineages, closely related to *M. bovis*. The lineages are strongly geographically associated. Nucleotide substitutions proven to cause drug resistance are distributed throughout the tree, whereas nonsynonymous base substitutions unrelated to drug resistance have a restricted distribution. The phylogenetic structure is concordant with all the previously described genotypic and phenotypic groupings of *M. tuberculosis* strains and provides a unifying framework for both epidemiologic and evolutionary analysis of *M. tuberculosis* populations.

Mycobacterium tuberculosis has caused tuberculosis (TB) in humans for thousands of years (1,2), and the World Health Organization (WHO) estimates that one third of the global population is infected with *M. tuberculosis* (3); however, the bacterium has remained an enigma. The global resurgence of TB highlights the need for an improved understanding of its epidemiology and its evolutionary biologic features. Recent advances in molecular characterization of *M. tuberculosis* isolates, which index variation in insertion sequences (4) and repetitive genomic elements (5,6), have elucidated clusters of identical and closely related strain families (7–9). These findings have provided insights into regional (10) and national (11) epidemiologic features. However, these techniques may be less suited to global population and evolutionary analyses, and integrating information obtained from different approaches is complex (12). Genomic comparisons have identified genetic variation for population screening; however, these analyses are limited to those sites that vary between the compared genomes and are potentially misleading (13–15). Nucleotide sequences provide robust, portable, and comparable data for studying population variation. The mutational processes that generate this variation are understood, and sequence data have been successfully used in the study of bacterial epidemiology, population biology, and evolution (16). The complete genome sequences (15–18) provide access to all regions of the chromosome and facilitate such studies. However, high-throughput gene sequencing of structural genes (19) and host immune system protein targets (20) in *M. tuberculosis* isolates indicated low levels of sequence diversity. Although extensive genomic sequencing was performed in both studies, comparable sequence data were obtained on a limited number of highly selected isolates.

We used an unbiased population approach to analyze genetically silent nucleotide sequence variation for seven unlinked loci distributed around the chromosome. The loci chosen were genes associated with antimicrobial drug resistance that have been reported to possess >95% of all sequence variation observed in 26 structural genes studied (19), which includes >90% of synonymous nucleotide substitutions, i.e., nucleotide substitutions that do not affect the translated amino acid. In a population sample of 316 U.K. clinical isolates, silent single nucleotide polymorphisms (sSNPs) resolve an unambiguous phylogeny and provide a unifying framework for epidemiologic, population, and evolutionary analyses.

Methods

Bacterial Strains

The 316 *M. tuberculosis* clinical isolates were identified in England and Wales from January 1, 1998, through

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December 31, 1998, and included all the viable clinical isolates (n = 216) resistant to one or both of the firstline antituberculous drugs (rifampicin and isoniazid) and 100 randomly chosen fully susceptible isolates. One *M. tuberculosis* H37Rv isolate, four *M. bovis* isolates, two *M. africanum* type I isolates, and one *M. microti* isolate were included for comparison. *M. tuberculosis* complex isolates were identified with a combination of microscopic and macroscopic appearance, growth characteristics, biochemical analysis, and DNA hybridization (21). Clinical and epidemiologic information were obtained from laboratory records at the HPA Mycobacterium Reference Unit, London, UK, the U.K. Mycobacterial Resistance Network database (MYCOBNET), and the 1998 national TB survey (22).Duplicate isolates were excluded. Drug susceptibility was determined by the resistance ratio method (2). Strains were characterized by IS*6110* restriction fragment length polymorphism (RFLP) and spoligotyping (4,5)

Amplifying and Sequencing Target Gene Loci

The nucleotide sequences were obtained for the following seven gene loci: *rpoB*, *katG*, *oxyR*, *ahpC*, *pncA*, *rpsL*, and *gyrA*. These gene loci are associated with drug resistance, but without antimicrobial drug selection pressure, they would be regarded as housekeeping genes. Primers and amplification conditions for polymerase chain reaction (PCR) are shown in Table 1. Products were purified by precipitation with 20% polyethylene glycol–2.5 mol/L NaCl and sequenced from both DNA strands by using internal nested primers (Table 2) and BigDye Ready Reaction Mix (ABI, Warrington, UK) according to the manufacturer's instructions. Unincorporated dye terminators were removed by precipitation with 96% ethanol–0.115 mol/L sodium acetate, pH 4.6. The reaction products were separated and detected with an ABI prism 3700.

Genetic Analysis

Sequences were assembled with the STADEN suite of computer programs (23). The sequences were compared, and isolates with identical sequences were assigned the same allele number. For each gene, the DNA sequence was translated in frame, and each nucleotide polymorphism characterizing the allele was classified as synonymous or genetically silent, nonsynonymous, or intergenic.

For each isolate, the concatenated sequences from the coding region of all seven gene loci were reduced to a 36-nt sequence motif, constituting a synonymous sequence profile (SSP), and distinct SSPs were assigned a synonymous sequence type (SST). Phylogenetic analysis of the SST motifs was performed with the MEGA (24) and PHYLIP software packages (25): 225 isolates were sequenced at all loci. The sequencing data from the initial 225 isolates demonstrated key polymorphisms that were lineage defining. The remaining 94 isolates were assigned a lineage based on polymorphisms at *katG*^{87}, *katG*^{609}, *katG*^{1388}, *oxyR*^{37}, *oxyR*^{285}, and *ahpC*$^{-46}$, and by the spoligotype deletion pattern. A lineage was only ascribed if all data points agreed (Table 3). The relationship between lineages, phenotypic and genotypic drug resistance, and country of birth was analyzed with chi-square and Fisher exact test.

Comparison with Outgroups

An in silico analysis of the seven gene loci was undertaken for two mycobacterial outgroups, *M. leprae* (26) and *M. marinum* with BLAST (Sanger Institute, Cambridge, UK; available from http//www.sanger.ac.uk/projects/M_marinum/). The complete gene sequences for each of the seven loci in *M. tuberculosis*, *M. bovis*, *M. leprae*, and *M. marinum* were aligned in frame by using Clustal-W. Two approaches were used. First, the aligned sequences for the coding regions of the seven gene loci were concatenated to produce a single sequence of 8.212 Kbp for each isolate. The concatenated sequences for fully susceptible examples of the *M. tuberculosis* SSTs were aligned to this. Second, SSPs were constructed for *M. leprae* and *M. marinum* by using the relevant aligned nucleotide for each of the previously identified variable synonymous sites in *M. tuberculosis* and *M. bovis*. For each approach, a phylogeny was constructed with the neighbor-joining tree method, and the results were compared.

TbD1-PCR Analysis

Three isolates per SST were selected for analysis. Each possessed, when possible, a different IS*6110* RFLP or spoligotype pattern. This analysis was performed with the published method (13).

Results

Observed Genetic Diversity

The complete gene was sequenced for all but one locus, which provided 8,318 Kbp of nucleotide sequence data for each isolate. Across the seven loci, 115 variable sites were identified, of which 101 were within the coding region of the selected loci, and 36 were associated with genetically neutral base substitutions. The number of alleles per locus varied from 6 (*oxyR*) to 40 (*rpoB*). The proportion of variable sites present at each locus was low, 0.68% (*rpoB*) to 2.68% (*pncA*). Nonsynonymous base substitutions were more frequent than synonymous substitutions at almost all loci. The ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site (d_N/d_S ratio) varied from 0.109 to 0.848 in sensitive *M. tuberculosis* isolates and from 0.301 to 1.952

Table 1. Amplification primers

Gene/locus	Forward primer	Reverse primer	Product (bp)	Reaction conditions[a]	Cycles
gyrA	gyrA-ext F 5′-ACAGACACGACGTTGCCGCC-3′	gyrA-ext R 5′′-GTCGATTTCCCTCAGCATCTCC-3′	435	95°C for 15 min[b] 95°C for 15 s 68°C for 30 s 72°C for 1 min 72°C for 5 min	30
inhA	mabA-ext F 5′-TCGTAGGGCGTCAATACACC-3′	mabA-ext R 5′-TCATTCGACCGAATTTGTTG-3′	605	94°C for 5 min 94°C for 30 s 60°C for 30 s 72°C for 30 s 72°C for 5 min	30
katG	katG-ext3F 5′-CGACGAAATGGGACAACAGT-3′	katG-ext3R 5′-TGCATGAGCATTATCCCGTA-3′	1,507	94°C for 5 min 94°C for 30 s 60°C for 30 s 72°C for 1 min 72°C for 7 min	30
	katG -ext5F 5′-TCGACTGTGCTGTTGGCGAGG-3′	katG-ext5R 5′-CTTCGCCGACGAGGTCGTGG-3′	1,531	95°C for 15 min[b] 95°C for 30 s 68°C for 30 s 72°C for 1 min 72°C for 7 min	30
oxyR-ahpC	oxyR-ext F 5′-TCGAGCTGCGACGGTGCTGG-3′	oxyR-extR 5′-CTGCGGGTGATTGAGCTCAGG-3′	1,437	95°C for 15 min[b] 95°C for 30 s 72°C for 30 s 72°C for 1 min 72°C for 7 min	30
pncA	pncA-ext F 5′′-AACCAAGGACTTCCACATCG-3′	pncA-extAR 5′-CAGAAACTGCAGCATCATCG-3′	1,324	95°C for 15 min[b] 95°C for 30 s 64°C for 30 s 72°C for 1 min 72°C for 7 min	30
rpoB	rpoB-46F 5′-GGCCGTGGGCACCGCTCC-3′	rpoB 1868R 5′-CCAGCGGGGCCTCGCTACG-3′	1,822	95°C for 15 min[b] 95°C for 15 s 65°C for 30 s 72°C for 3 min 72°C for 10 min	30
	rpoB 1711F 5′-GTGCCCTCGTCTGAGGTGGAC-3′	rpoB 3602R 5′-AAGACCGATGCGGAGTTCATCG-3′	1,891	95°C for 15 min[b] 95°C for 15 s 65°C for 30 s 72°C for 3 min 72°C for 10 min	30
rpsL	rpsL-extF 5′-GGCCGACAAACAGAACGT-3′	rpsL-extR 5′-GTTCACCAACTGGGTGAC-3′	494	94°C for 5 min 94°C for 30 s 56°C for 30 s 72°C for 30 s 72°C for 5 min	30

[a]A final PCR reaction volume of 25 µL was used that contained 2.5 µL of 10 x ammonium sulfate reaction buffer (Bioline, London, UK), 1.5 mmol/L magnesium chloride (Bioline); 200 µmol/L each of dATP, dTTP, dGTP, and dCTP;, 300 nmol/L of each primer pair, 0.8 units of Taq DNA polymerase (Bioline), 1 µL (≈10 ng) of template DNA and sterile distilled water. Amplification was carried out in 0.2 ml thin-wall polymerase chain reaction tubes in a DNA Thermal Cycler 9600 (Applied Biosystems, Warrington, UK). Products were purified by precipitation with 20% polyethylene detected with an ABI Prism 3700 or an ABI Prism 377 automated DNA sequencer (ABI, Warrington, UK).
[b]Reaction performed with Hotstar Taq and reaction buffer (Qiagen, Crawley, UK).

overall, which implied that resistance to antituberculous medication is indeed the selective force at most loci. Five variable sites were unique to *M. bovis*, of which four were associated with synonymous polymorphisms. A further variable site with a previously reported synonymous polymorphism (*katG* ^{C609T}) (27) was identified in both *M. bovis* and *M. microti*, present in all four *M. bovis* isolates sequenced and the published *M. bovis* genome sequence (18).

Synonymous Sequence Types and Lineages

By disregarding nonsynonymous polymorphisms, i.e., those producing an amino acid change likely in response to diversifying or stabilizing selection, a subset of 37 neutral sSNPs at 36 sites was generated; one site possessed two different synonymous substitutions. These substitutions occurred in 35 unique combinations, which we term synonymous sequence types (SSTs); each was assigned an arbitrary number (Figure 2). The variation in the SSTs conformed to the clonal model for bacterial population

Table 2. Sequencing primers[a]

Locus/PCR product	Forward primers	Reverse primers
gyrA	gyrA-1F 5′-CAGCTACATCGACTATG-3′	gyrA-1R 5′-GGGCTTCGGTGTACCTCAT-3′
inhA promoter	mabA-1F 5′-AGAAAGGGATCCGTCATGGT-3′	mabA-1R 5′-GTCACATTCGACGCCAAAC-3′
katG 3F-3R	katG-1F 5′-ACGCGGGGTCTGACAAAT-3′	katG-1R 5′-GACAAGGCGAACCTGCTTAC-3′
	katg-2F 5′-GTAAGCAGGTTCGCCTTGT-3′	katG-2R 5′-TCGGGATTGACTGTCTCACA-3′
	katG-3F 5′-ATCTCTTCCAGGGTGCGAAT-3′	katG-3R 5′-GAGTGGGAGCTGACGAAGAG-3′
katG 5F-5R	katG-4F 5′-AGAGGTCAGTGGCCAGCAT-3′	katG-4R 5′-AGATGGGGCTGATCTACGTG-3′
	katG-5F 5′-GCTGTTTCGACGTCGTTCAT-3′	katG-5R 5′-ACTACGGGCCGCTGTTTATC-3′
	katG-6R 5′-ACACTTCGCGATCACATCC-3′	katG-6R 5′-ACACTTCGCGATCACATCC-3′
oxyR-ahpC	oxyR–1 5′-CTGGCCAGGTAAGACGACC-3′	oxyR-2 5′-CAGACGCTCGATGCTGCC-3′
	oxyR-7 5′-TCATATCGAGAATGCTTGCGG-3′	oxyR-4 5′-TGCTTGGCGTCCACCTTGG-3′
	oxyR-6 5′-TGATGTCTTTGGCGTACTCGG–3′	oxyR-6 5′-CAATGACGAGTTCGAGGACC-3′
pncA	pncA-P1 5′-GCTGGTCATGTTCGCGATCG-3′	pncA-R 5′-CGATGAAGGTGTCGTAGAAGC-3′
	pncA –F 5′-AACCAAGGACTTCCACATCG–3′	pncA-2F 5′-ATACCGACCACATCGACCTC-3′
rpoB–46-1868	rpoB –41F 5′-GTGGGCACCGCTCCTCTAAGG-3′	rpoB 509R 5′-TGACCACCACACGCTCGGTCC-3′
	rpoB 331F 5′-CGTTTCGACGATGTCAAGGCA-3′	rpoB 975R 5′-GTCGACGACGTGATGGGCTCG-3′
	rpoB 783F 5′-CTGGAGAAGGACAACACCGTCG-3′	rpoB 2 5′-GCACGTCGCGGACCTCCAGCC-3′
	rpoB 1 5′-GGTCGGCATGTCGCGGATGGA-3′	rpoB 1845R 5′-CGCTACGGACCAGCGGCACC-3′
rpoB 1711-3602	rpoB 1725F 5′-GGTGGACTACATGGACGTCTC-3′	rpoB 2313R 5′-GTCGGAGATGTTCGGGATGTCG-3′
	rpoB 2134F 5′-GAGATGGCGCTGGGCAAGAAC-3′	rpoB 2770R 5′-TCTGGCCGATGTTCATCCGTCG-3′
	rpoB 2600F 5′-AGCTGGTGCGTGTGTATGTGG-3′	rpoB 3213R 5′-GGCCTGCATGCCCCAGCACTCC-3′
	rpoB 3013F 5′-CCGTTCCCGTACCCGGTCACG-3′	rpoB 3581R 5′-GAAGAAGTTGACGTCGAGCAC-3′
rpsL	rpsL F 5′-ACGTGAAAGCGCCCAAGATAGA -3′	rpsL R 5′-ACCAACTGCGATCCGTAGACC-3′

[a]All sequencing reactions were performed in 96-well plates (Abgene, UK) in a DNA Thermal Cycler 9600 (Applied Biosystems, Warrington, UK) by using the following thermocycling conditions: 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 2 min.

structure (28), and the maximum parsimony method generated a phylogenetic tree with no homoplasies, i.e., the lack of independent occurrence of a polymorphism in more than one branch of the tree (Figure 1A, 2). Each branch corresponds to a unique combination of sSNPs. The phylogeny was robust, whether constructed with or without outgroup SSTs generated from the *M. leprae* and *M. marinum* genome sequences. The analysis identified four prominent *M. tuberculosis* lineages (numbered I to IV); the *M. bovis* isolates formed an additional lineage. The lineages are defined by distinct combinations of sSNPs (Figure 2). Virtually all of the nodes in the tree are occupied; internal nodes tend to be represented more frequently in the isolate population. Although a number of evolutionary scenarios are possible, the most likely explanation for this observation is that the sSNP variation arose recently.

Within the *M. tuberculosis* complex, the sSNPs clearly distinguish *M. tuberculosis* from *M. bovis* and *M. microti*, with the *M. microti* SST forming a node on the *M. bovis* lineage. The *M. africanum* type 1 isolates sequenced could not be distinguished from *M. tuberculosis* because they share SST-1.

SST Phylogeny and Population Subdivisions

A variety of approaches have been used previously to subdivide *M. tuberculosis* strains into definable groups. These include assignments based on two nonsynonymous polymorphisms in *katG* and *gyrA* (19); the presence or absence of a TB-specific genomic region of difference, TbD1 (13); variation within the genomic direct repeat region demonstrated by spoligotyping; and strain families defined by highly conserved DNA fingerprint patterns obtained by RFLP of the insertion element IS*6110*. Each technique defines a limited number of distinct subdivisions, which although different, overlap when techniques are compared. The sSNP phylogenetic tree was congruent with all of the previously described subdivisions (Figure 1).

katG and *gyr*A Polymorphisms

M. tuberculosis isolates can be divided into three groups (1–3) based on two apparently unselected nonsynonymous SNPs, *katG*$^{G\ 1388\ T}$, and *gyrA*$^{G\ 284\ C}$ (19). Group 1 is defined by the combination of *katG* $^{1388\ T}$ and *gyrA* $^{284\ C}$, group 2 by *katG* $^{1388\ G}$ and *gyrA* $^{284\ C}$, and group 3 by *katG* $^{1388\ G}$ and *gyrA* $^{284\ G}$. We characterized the *katG*-*gyrA* polymorphisms in all isolates. The *katG* $^{G\ 1388\ T}$ polymorphism cosegregated in all cases with the synonymous base substitution *rpoB* $^{T\ 3243\ C}$ found in lineages I, III, IV, and *M. bovis*, whereas the *gyrA* $^{G\ 284\ C}$ polymorphism subdivided SST-2 in lineage II. Group 1 isolates can therefore be subdivided into three prominent *M. tuberculosis* lineages (I, III, and IV) made up of 21 SSTs and *M. bovis*. Group 2 and 3 *M. tuberculosis* isolates are subdivisions of lineage II. Group 2 isolates can be further subdivided into 10 SSTs, whereas group 3 isolates are confined to a subdivision of SST 2 (Figure 1B). When the SST of the clinical isolate

CDC1551 was identified in silico by analysis of the relevant sequences (www.tigr.org), it shared the same sequence type, SST 2, as the other isolate for which a complete genome is available, H37Rv. Although these two isolates are distinguished by numerous other synonymous and nonsynonymous polymorphisms (15), these organisms are closely related, which has implications for genetic variation based on comparing the two complete genome sequences (14,29).

TbD1 Region of Difference

The presence or absence of DNA regions, identified by genomic comparisons of *M. tuberculosis* H37Rv and *M. bovis* BCG, can be used to distinguish the closely related members of the *M. tuberculosis* complex. However, only two groups of *M. tuberculosis* isolates have been described with this approach, defined by the presence or absence of the TB-specific region, TbD1 (13). TbD1 PCR analysis was performed on three epidemiologically unrelated isolates from each SST, when available. We found that the TbD1 region was present in all 13 SST types constituting lineage IV and all *M. bovis*, *M. microti*, and *M. africanum* type I isolates, but the region was absent from all other lineages and SSTs (Figure 1C). This finding implies that the TbD1 deletion occurred before both the *katG* $^{G\ 1388\ T}$ and the *rpoB* $^{T\ 3243\ C}$ mutations. Although SST-1 appears the least differentiated SST with the maximum parsimony method and sSNP data alone, when taken together with the TbD1 data, the ancestors of lineage IV are likely to have diverged from *M. africanum* type I, *M. microti*, and *M. bovis* before differentiation of the other *M. tuberculosis* lineages.

DNA Fingerprinting Techniques

Molecular epidemiologic analyses of TB populations use a combination of typing techniques, most commonly IS*6110* RFLP analysis and spoligotyping. Spoligotyping has been used to distinguish members of the *M. tuberculosis* complex (5,30), and together with IS*6110* RFLP, has been used to describe various *M. tuberculosis* strain families including Beijing (7), Haarlem (8), Africa (8), Delhi (9), East Africa-India (EA-I), and Latin America-Mediterranean (12). Both techniques were used to type all 316 isolates, producing 234 IS*6110* RFLP patterns and 263 spoligotyping patterns; 157 isolates were assigned to strain families.

All isolates within each family were confined to a single lineage (Figure 1D). Furthermore, each lineage was defined by a distinct pattern of spacer deletions (Figure 1E). The signature spoligotype spacer deletion pattern for lineage II (lack of probe hybridization at spacers 33–36) concurs with that previously noted in group 2 and group 3 *M. tuberculosis* isolates (13,31). Lineage I was defined by the signature spoligotype of the Beijing family (absence of spacers 1–34), and lineage IV by the signature spoligotype of the EA-I family. The remaining strain

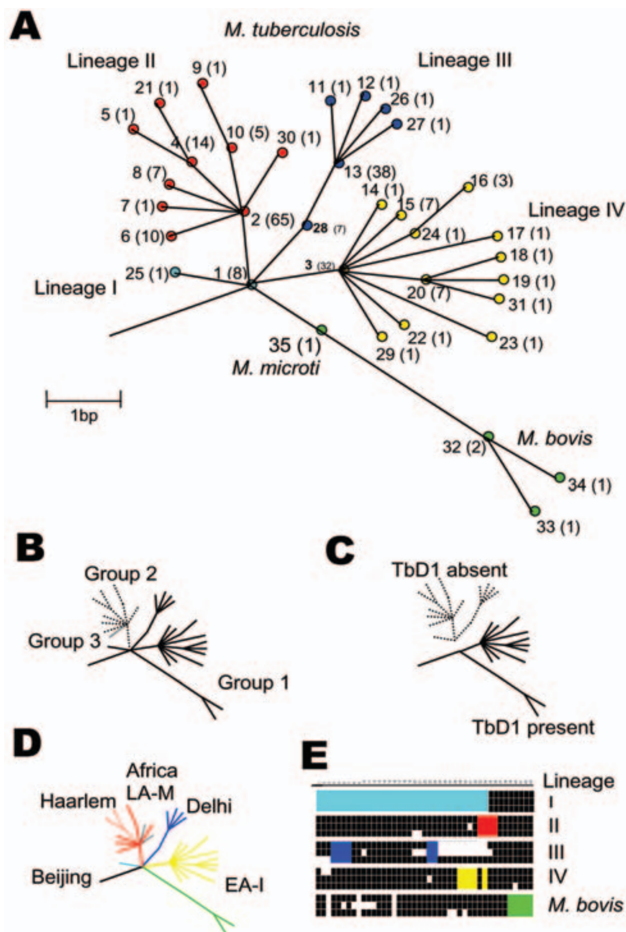

Figure 1. Unifying phylogeny for *Mycobacterium tuberculosis*. A) Maximum parsimony tree of *M. tuberculosis* and *M. bovis* based on 37 silent single-nucleotide polymorphisms in 225 isolates. Synonymous sequence types (SST) are marked 1–35. The frequency of each SST is marked in parentheses. The nodes of the major lineages are highlighted: lineage I (cyan), lineage II (red), lineage III (blue), lineage IV (yellow), and M. bovis (green). The colors correspond to those in Figure 2. Note both *M. africanum* Type I isolates sequenced were SST 1. B) Schematic representation of the genetic groups 1, 2, and 3 defined by the *katG-gyrA* scheme. C) Schematic representation of the presence or absence of the tuberculosis specific region of difference, TbD1. D) Schematic representation of the strain families Beijing, Haarlem, Africa, Delhi, East Africa-India (EA-I), and Latin America-Mediterranean (LA-M), previously described by IS*6110* restriction fragment length polymorphism typing and spoligotyping, demonstrating concordance with the phylogenetic tree. E) Spoligotyping patterns for representative isolates of each lineage demonstrating lack of probe hybridization at spacers 1–34 in lineage I, 33–36 in lineage II, 4–7 and 23–24 in lineage III, 29–32 and 34 in lineage IV, and 39–43 in *M. bovis.*

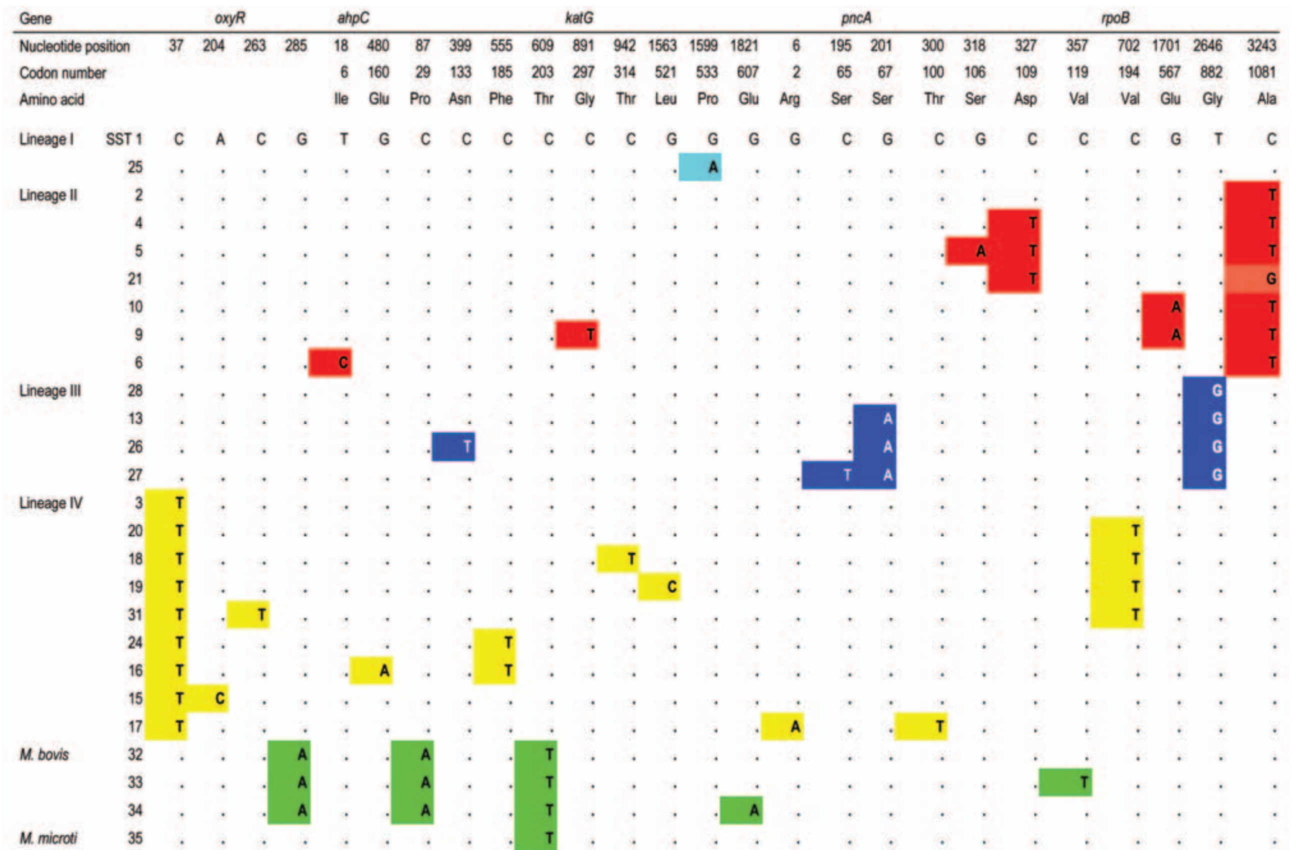

Figure 2. Abbreviated figure demonstrating the relationship between the silent single nucleotide polymorphisms, the synonymous sequence type, and the major lineages (abbreviated to 26 SSTs and 26 variable sites. •, represents invariant base with respect to SST 1. For full reproduction of this figure, please see http://www.cdc.gov/ncidod/eid/vol10no9/04-0046-G2.htm).

families were confined to lineage subbranches, including the Haarlem family, confined to SSTs 4, 5, and 6 of lineage II, and the Delhi family, confined to lineage III, almost exclusively within SSTs 11, 12, 13, 26, and 27. The relationship between SST, lineage, spoligotype pattern, and IS*6110* RFLP pattern are shown in more detail in online Figure available at http://www.cdc.gov/ncidod/eid/vol10no9/04-0046-G2.htm.

Seventy-one isolates shared identical RFLP and spoligotype patterns with one or more isolate, grouped in 23 clusters, of which 10 possessed RFLP patterns containing five or fewer IS*6110* copies. Cluster sizes ranged from two to seven isolates, with a median cluster size of two. Isolate clusters were present in all lineages, but isolates with low copy number were confined to lineage II and IV. Three of the low copy number clusters, each characterized by a single IS*6110* band and distinct spoligotype pattern, were subdivided by SST, whereas among high copy number clusters, all isolates within a cluster possessed the same SST. To prevent introducing a selection bias, no correction was made for strain transmission.

Polymorphisms and Antimicrobial Drug Resistance to Lineage

Having demonstrated a robust phylogeny for *M. tuberculosis* on the basis of neutral genetic variation, we annotated the tree with the nsSNPs. Most nsSNPs were rare within the isolate collection examined, many of which were represented uniquely. However, a number of nsSNPs known to confer drug resistance occurred frequently, including *rpoB* $^{C\ 1367\ G}$, *rpoB* $^{C\ 1367\ T}$, *rpoB* $^{C\ 1351\ T}$ (32,33), *katG* $^{A\ 944\ C}$ (34,35), *rpsL* $^{A\ 128\ G}$ (36), and *inhA* $^{C-15\ T}$ promoter mutation (35). These polymorphisms were distributed throughout the phylogeny, which implies that they arose independently on many occasions, presumably in response to the positive selection imposed by antimicrobial drug use. In contrast, an intergenic SNP, *oxyR-ahpC* $^{G\ -46\ A}$ (37), associated with, but not proven to cause, isoniazid resistance, occurred exclusively in lineage III and was present in all isolates within the lineage, which implies that this SNP may have arisen under neutral selection.

Although mutations conferring drug resistance were present in all lineages, the proportion of resistant and susceptible isolates varied between lineages. When antimicro-

Table 3. Characteristic features of the four major lineages of *Mycobacterium tuberculosis* with respect to *M. bovis*

Species/lineage	TbD1 region of difference	Silent single nucleotide polymorphism: *rpoB* 3243 T	*rpoB* 2646 G	*katG* 87 A	*katG* 609 T	*oxyR* 285 A	*oxyR* 37 T	Nonsynonymous base substitutions: *katG* 1388 G	*ahpC* –46 A	Spoligotype signature spacer deletion
M. tuberculosis Lineage I	–	–	–	–	–	–	–	–	–	1–34
M. tuberculosis Lineage II	–	+	–	–	–	–	–	+	–	33–36
M. tuberculosis Lineage III	–	–	+	–	–	–	–	–	+	4–8, 23–24
M. tuberculosis Lineage IV	+	–	–	–	–	–	+	–	–	29–32 and 34
M. bovis	+	–	–	+	+	+	–	–	–	39–43

bial drug susceptibility data were used, lineage III was positively associated with isoniazid resistance (51/62, p = 0.002), Lineages I and III were positively associated with streptomycin resistance (12/20, p = 0.0004 and 24/62, p = 0.004 respectively), and lineage IV was positively associated with fully susceptible isolates (30/62, p = 0.002). Rifampicin resistance was not associated with any lineage.

Genotypic analysis of phenotypically resistant isolates showed that among isoniazid-resistant isolates, the resistance-conferring mutation *katG* $^{A\,944\,C}$ was positively associated with lineage III (odds ratio [OR] 2.44, p = 0.016) and the *inhA* $^{C\,-15\,T}$ promoter mutation positively associated with lineage IV (OR 3.28, p = 0.006). Among streptomycin-resistant isolates, the resistance-conferring mutation *rpsL* $^{A\,128\,G}$ was positively associated with lineage I (OR 7.83, p = 0.012) and negatively associated with lineage II (OR 0.32, p = 0.036). No genotype-lineage association was identified for rifampicin resistance. Different lineages have significant differences in their antimicrobial drug susceptibility to certain antimicrobial agents, perhaps demonstrating the effect of genomic environment on the probability of mutational events conferring resistance to these antimicrobial drugs.

Lineage to Country of Birth

Drug resistance in countries with low TB incidence has been associated with foreign-born migrants (11); 44% of TB cases in England and Wales occur in the indigenous population (22). Information about country of birth was available for 225 (71%) of the patients; they represented 45 countries. Foreign-born patients had a median residency of 4 years in the United Kingdom. There was no significant difference between patients infected with susceptible or drug-resistant *M. tuberculosis* with respect to patient country or continent of birth. However, highly significant associations existed between continent of birth and lineage (Table 4). Lineages I, II, and III were significantly associated with southeastern Asia, Europe, and the Indian subcontinent, respectively. Lineage IV, in contrast, was globally distributed but had a negative association with Europe. This finding provides strong evidence for geographic structuring in *M. tuberculosis* populations.

Discussion

Synonymous nucleotide polymorphisms reflect neutral genomic variation, which remains informative, even in genes that have recently experienced positive selection attributable to introducing antimicrobial agents. By sequencing widely at multiple gene loci around the chromosome in a population sample of *M. tuberculosis* isolates, selection bias is avoided and all neutral variation within the sequenced regions will be identified. The indexed variation is highly unlikely to arise by convergence, which provides a robust base for constructing a phylogenetic tree. Our data support the belief that *M. tuberculosis* is a strictly clonal organism, with no evidence of lateral gene transfer.

Individual sSNPs are confined to clonally related organisms and accumulated by subsequent generations. Each of the lineages defined here can be defined on the basis of

Table 4. Relationship between *Mycobacterium tuberculosis* lineage and continent of birth of patient[a]

Lineage	n	Europe: Eur	Not Eur	OR	p value	Africa: Afr	Not Afr	OR	p value	Indian subcontinent: ISC	Not ISC	OR	p value	Southeast Asia: SEA	Not SEA	OR	p value
I	17	5	12	0.86	0.997	1	16	0.17	0.079	1	16	0.12	0.034	10	7	28.8	< 0.00001
II	118	59	59	6.4	< 0.00001	33	85	1.34	0.473	19	99	0.2	< 0.00001	4	114	0.21	0.006
III	50	3	47	0.1	0.00001	9	41	0.58	0.245	37	13	11.31	< 0.00001	0	50	0.0	0.009
IV	40	5	35	0.25	0.005	15	25	2.04	0.08	15	25	1.36	0.514	5	35	1.66	0.166
Total	225	72	153			58	167			72	153			19	206		

[a]OR, odds ratio; Eur, Europe; Afr, Africa; ISC, Indian subcontinent; SEA, southeast Asia.

single sSNPs, yet the resultant maximum parsimony tree provides a robust and unifying phylogeny for *M. tuberculosis*. The documented population diversity is relatively recent, demonstrated by SSTs that represent almost all of the phylogenetic nodes. In contrast, *M. tuberculosis* and *M. bovis* separated from a common ancestor more distantly, which reinforces the evidence that *M. tuberculosis* could not have arisen from *M. bovis*, as previously thought.

Although we have sequenced a small number of *M. tuberculosis* complex isolates, our data support the evolutionary scenario described by Brosch et al. (13). *M. microti* is a subdivision of the *M. bovis* lineage, diverging after the separation of *M. bovis* from its common ancestor with *M. tuberculosis. M. africanum* type I, which cannot be distinguished from *M. tuberculosis* on the basis of neutral variation in the genes sequenced in this study, can be distinguished from *M. tuberculosis* SST-1 by the presence of the TbD1 region. Taken together, the sequence data support divergence of *M. africanum* type I from a common ancestor with *M. tuberculosis* before the subsequent divergence of *M. microti* and *M. bovis*. Analyzing silent nucleotide polymorphisms in *gyrB,* which has been used to distinguish members of the *M. tuberculosis* complex (38,39), would provide further neutral sequence variation to support the evolutionary scenario.

M. tuberculosis isolates in England and Wales represent four clearly defined lineages. Although the isolates were all obtained from patients residing in the United Kingdom, the patients represented 45 countries of birth, from four continents. The population is not globally representative; for example, few patients originated from the Americas. Nevertheless, the strong geographic structuring of the *M. tuberculosis* population is striking. *M. tuberculosis* is an obligate human pathogen, with a delay between initial infection and the development of clinical disease (often up to 5 years) and long periods of latency between disease control and subsequent clinical reactivation. The evolution and global dissemination of *M. tuberculosis* are by definition associated with the activities of its human host. Although foreign-born patients may have been infected with *M. tuberculosis* in the United Kingdom, the short median residency in the United Kingdom and the lack of strain clustering support the hypothesis that these are imported strains reflecting *M. tuberculosis* populations in the patient's country of birth. Clonal expansion of geographically restricted, genetically distinct lineages presumably reflects the previously geographically limited human population movements, with higher rates of transmission within, rather than between, geographic regions. No single *M. tuberculosis* lineage dominates in African-born patients. As in human populations, Africa appears to be a melting pot for genetic diversity. This fact may reflect the dissemination of *M. tuberculosis* by ancient human migration and trade routes but could be further elucidated by analysis of unselected isolates obtained in Africa.

Unlike lineages I, II and III, lineage IV is globally distributed, with no discernible geographic association. Not only is it the only lineage possessing the TbD1 region of difference, in common with *M. bovis*, *M. microti*, and *M. africanum* type I (13), but a large proportion of the isolates possess only a single IS*6110* copy, and isolates from the lineage are negatively associated with antimicrobial drug resistance. These data suggest that *M. tuberculosis* isolates from lineage IV are more closely related to the common ancestor of the *M. tuberculosis* complex, unexposed to antimicrobial selection pressure, and provide evidence to support the hypothesis that *M. tuberculosis* isolates possessing a single IS*6110* copy may be ancestral (40,41). In contrast, isolates possessing a high number of IS*6110* copies are present in all four *M. tuberculosis* lineages, which reflects independent IS*6110* transposition events in different parts of the phylogeny.

Geographic structuring of a clonal population will result in genetically and phenotypically distinct *M. tuberculosis* populations, which may explain, in part, the geographically variable response to vaccination with *M. bovis* BCG, or striking differences in clinical features, such as the predominance of extrapulmonary disease in patients originally from the Indian subcontinent. This finding may also have implications for the successful development of new TB vaccines. Nucleotide substitutions arising under neutral, positive, and negative pressure will all become fixed, inherited by all clonal descendants. Analyzing mutations that confer antimicrobial drug resistance provides an insight into this evolutionary process. By definition, resistance-conferring mutations are associated with phenotypic resistance absolutely. The genes involved all encode essential metabolic functions, restricting nonsynonymous nucleotide substitutions. The data demonstrate that the most frequently reported resistance-conferring mutations are present in all lineages, which implies that they have arisen independently on multiple separate occasions; however, phenotypically antimicrobial drug resistance is significantly associated with lineage. The significantly greater proportion of phenotypically resistant isolates with the *katG* ^{315} mutation in lineage III and the observation that the mutation is not present in all isolates within clonally related subbranches of the tree confirms the relatively recent influence of antimicrobial drug selection pressure. This finding implies that isolates within the lineage may be biochemically more susceptible to acquiring the same resistance conferring mutation. Rifampicin resistance and multidrug-resistant TB isolates were unrelated to lineage, although the numbers were relatively small (52 phenotypically rifampicin-resistant isolates, of which 46 were multidrug resistant).

We have shown that the sequenced *M. tuberculosis* strains, CDC1551 and H37Rv, are closely related and come from the same major lineage (lineage II, SST-2). This relation has implications for sSNP analyses based on comparison of only these genomes, collapsing subbranches and skewing any resultant phylogenetic tree (14,29). Although this tendency can be reduced slightly by comparing genomes that are genetically more divergent, the lack of horizontal gene transfer in a clonal population means that variation in other branches of the phylogeny will not be revealed. In fact, only four of the sSNPs described here can be resolved by genome comparison of the four completed genome sequences *M. tuberculosis* H37Rv (17), CDC1551 (15), strain 210 (www.tigr.org), and *M. bovis* (18). None were used in the SNP analysis performed by Gutacker et al. (14). By sequencing widely at multiple gene loci around the chromosome, we have identified all the indexable genetically neutral variation (sSNPs) within the sequenced regions. Although Sreevatsan et al. used a similar approach in their study of 26 structural genes, which included regions of all seven genes sequenced in this study (19), the isolates were selected from a large collection of *M. tuberculosis* strains in part on diversity in IS*6110* RFLP (introducing a bias towards high copy number strains), and the number of isolates sequenced at each locus varied, with no defined minimum dataset. We identified a similar level of genomic sequence diversity, but by using an unbiased population approach, we have shown phylogenetically significant neutral variation.

The phylogeny described here is unambiguous and can be defined with a limited number of sSNPs. These could easily be identified with rapid screening techniques. Simultaneous identification of nsSNPs associated with antimicrobial drug resistance would provide data valuable for clinical, epidemiologic, and evolutionary purposes in a single, cost-effective, and highly portable format that is amenable to electronic database comparisons (16).

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Genetic Divergence and Dispersal of Yellow Fever Virus, Brazil

Pedro F.C. Vasconcelos,* Juliet E. Bryant,† Amelia P.A.Travassos da Rosa,† Robert B. Tesh,† Sueli G. Rodrigues,* and Alan D.T. Barrett†

An analysis of 79 yellow fever virus (YFV) isolates collected from 1935 to 2001 in Brazil showed a single genotype (South America I) circulating in the country, with the exception of a single strain from Rondônia, which represented South America genotype II. Brazilian YFV strains have diverged into two clades; an older clade appears to have become extinct and another has become the dominant lineage in recent years. Pairwise nucleotide diversity between strains ranged from 0% to 7.4%, while amino acid divergence ranged from 0% to 4.6%. Phylogenetic analysis indicated traffic of virus variants through large geographic areas and suggested that migration of infected people may be an important mechanism of virus dispersal. Isolation of vaccine virus from a patient with a fatal case suggests that vaccine-related illness may have been misdiagnosed in the past.

Yellow fever virus (YFV) is transmitted by the bite of infected mosquitoes and produces a severe hemorrhagic fever in humans. Despite a safe and effective vaccine (17D), YFV continues to be a public health problem in tropical areas of Africa and South America (1–3). A recent upsurge in YFV activity in Brazil and the reinfestation of urban areas with the vector mosquito *Aedes aegypti* have stretched disease surveillance and control resources to their limits. During 2000, YFV occurred near Brasilia, the capital city (4); in 2001, YFV spread to new areas of Minas Gerais outside the currently recognized enzootic zone (5).

In South America, YFV is maintained in enzootic cycles involving monkeys and forest-canopy mosquitoes of the genera *Haemagogus* and *Sabethes* (6,7). In Brazil, three geographic zones have been defined where YFV circulates (7) (Figure 1): 1) the region of endemicity, in which the virus is maintained in mobile monkey populations and where human cases are sporadic and rare; 2) transitional zones of emergence, in which contact is frequent between monkey and human populations (and infected mosquito vectors); and 3) regions of epidemicity where the density of susceptible human populations and competent vector species are both high, and the potential for explosive urban outbreaks is great. Brazil currently has a population of about 176 million; approximately 30 million people live in the YFV-endemic zone, 18 million live in the zone of emergence, and 128 million reside along the Atlantic coast in the YFV-free zone (8,9).

Many aspects of the molecular epidemiology and transmission cycles of YFV in the forests of South America are poorly understood, and previous studies of YFV in South America were limited to a relatively small number of isolates. We examined the genetic diversity of 79 YFV strains isolated from Brazil over 67 years and mapped the distribution of variants to investigate patterns of virus divergence and dispersal.

Material and Methods

Study Area

Brazil is a country of enormous size and diversity, covering 8,512,000 km^2. The country is divided into 26 states and the Federal District. These make up five major geographic regions, characterized by broadly different climate and vegetation zones and a highly variable distribution of the human population. The five regions (Figure 1) consist of the northern Amazon region, northeastern region (*caatinga*, very dry), central-western region (swamp and savannah), southeastern region (most heavily populated with an extensive system of roads and railways), and the more temperate southern region bordering Argentina and Paraguay (10).

Virus Strains

Strain-specific data such as geographic locality, passage history, source of isolate, and clinical outcome (for

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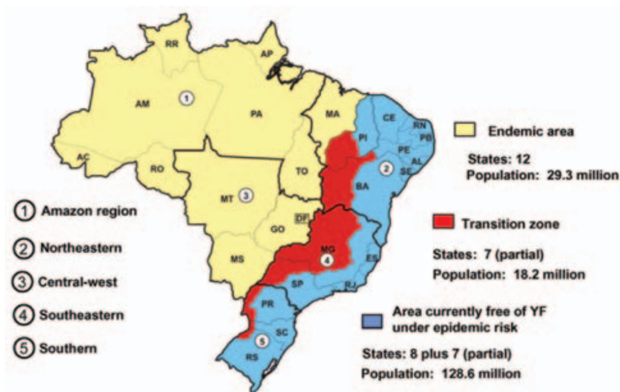

Figure 1. Regions where yellow fever is endemic in Brazil.

selected human cases) for the 79 Brazilian YFV strains used in this study are provided in the online Appendix (http://www.cdc.gov/ncidod/eid/vol10no9/04-0197_app.htm). All strains were originally isolated in suckling mice and cultured once in C6/36 cells to produce seed stocks (online Appendix) at the Instituto Evandro Chagas (IEC), Belém, Brazil. Methods for cell culture and virus growth have been previously described (11–13), and standard laboratory precautions within biosafety level 3 facilities were undertaken to prevent cross-contamination of strains. Thirty-eight (48%) virus strains were from humans (24 from patients who died); 7 (9%) were from monkeys; and 34 (43%) were from mosquito pools, mainly *Haemagogus janthinomys*. Viruses represented a period of 67 years, but with unequal sample distribution: 15% of strains were obtained from 1935 to 1969, 43% were obtained from 1970 to 1989, and 42% were obtained from 1990 to 2001. The year of virus isolation is indicated in the sequence identification (e.g., Brazil35, isolated in 1935). Isolates were from 12 states: Amapá (n = 1), Bahia (n = 1), Federal District (n = 1), Goiás (n = 10), Maranhão (n = 6), Minas Gerais (n = 7), Mato Grosso (n = 4), Mato Grosso do Sul (n = 5), Pará (n = 39), Rondônia (n = 2), Roraima (n = 1), and Tocantins (n = 2).

Phylogenetic Analysis

Supernatants of YFV-infected Vero cells were obtained, and viral RNA was extracted by using a commercial kit (Qiagen, Valencia, CA) and processed according to the manufacturer's instructions. RNA obtained was stored at –70°C. The genomic-sense degenerate primer EMF (5′ TGGATGACSACKGARGAYAT) and genomic-complementary primer VD8 (5′ GGGTCTCCTCTAACCTCTAG) were used for reverse transcription–polymerase chain reaction amplification of a 595-bp fragment comprising 255 nucleotides of NS5 and 340 nucleotides of 3′NCR (14,15). PCR products were screened by agarose gel electrophoresis. Bands were recovered with a gel extraction kit (Qiagen) and directly sequenced with an ABI automatic sequencer at the University of Texas Medical Branch protein chemistry core facility.

Sequence editing and alignments were performed with Vector NTI (Informax, Frederick, MD), and additional manual editing of alignments was performed with the GCG Wisconsin Package Version 10.3 (Accelrys, San Diego, CA). The PAUP* program (16) was used to infer phylogenetic trees by the neighbor-joining method with Kimura 2-parameter distance corrections. Support for individual clades was determined by nonparametric bootstrapping with 1,000 replicates. The tree was rooted with a sequence of the prototype West African YFV strain Asibi (17) and the 17DD substrain vaccine virus (18).

Results

Genetic Diversity of Brazilian YFV Strains

Sequence data were obtained for a genomic region spanning the terminal portion of NS5 and proximal region of the 3′NCR for 54 Brazilian YFV strains. In addition to the sequence data generated in this study, partial or complete 3′NCR sequences were available for an additional 25 Brazilian YFV isolates (13,14), which yielded the full dataset of 79 YFV strains from 12 states (alignment length, 576 nt). The NS5/3′NCR sequences contained 148 variable sites, of which 89 were informative. Thirty-two of the informative sites (36%) fell within the NS5 coding portion of the sequence, and 57 (64%) fell within the 3′NCR. Amino acid pairwise divergence among the partial NS5 sequences (85 amino acids in length) ranged from 0% to 4.7% (mean 2.2%).

A phylogenetic tree of the 79 Brazilian YFV sequences is shown in Figure 2. The tree is rooted with the homologous region of the Asibi prototype strain (parental virus to the 17D vaccine), isolated in Ghana in 1927 (17). Mosquito- and vertebrate-derived sequences were distributed randomly throughout the phylogenetic tree. With the exception of one 1983 isolate from Rondônia (BeH 413820) and one 1975 isolate from Aripuana in Mato Grosso (BeH 291597), all Brazilian YFV strains formed a single monophyletic clade. Two major subclades were evident: a subclade comprising isolates from Pará dating from 1954 to1968 and a subclade containing all remaining isolates from 1969 to 2001.

Variation within the Dominant Subclade

Genetic variation within the dominant subclade of Brazilian YFV strains showed a complex pattern of relationships that demonstrated both geographic and temporal associations. Varying levels of bootstrap support were evident for four clusters (groups 1A–1D) (Figure 2): group 1A included isolates from the 1972–1973 outbreak in

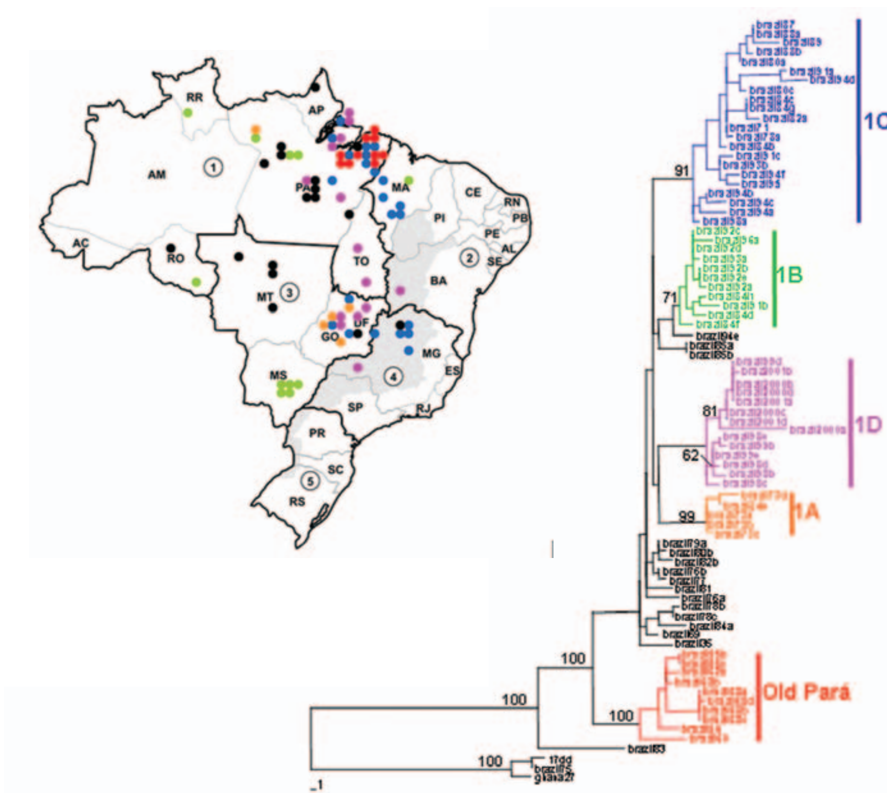

Figure 2. Brazilian NS5/3'NCR phylogeny (576 nt) based on yellow fever isolates (neighbor-joining tree, Kimura 2-parameter distance correction, midpoint rooted). Geographic origin of isolates is indicated on map. 1: North (AC, Acre; AM, Amazonas; AP, Amapá; PA, Pará; RO, Rondônia; RR, Roraima; TO, Tocantins). 2: Northeast (AL, Alagoas; BA, Bahia; CE, Ceará; MA, Maranhão; PB, Paraiba; PE, Pernambuco; PI, Piaui; RN, Rio Grande do Norte; SE, Sergipe). 3: Central West (DF, Distrito Federal; GO, Goiás; MT, Mato Grosso; MS, Mato Grosso do Sul). 4: Southeast (ES, Espírito Santo; MG, Minas Gerais; RJ, Rio de Janeiro; SP, São Paulo). 5: South (PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul). Colors correspond to genetic clade structure. Black dots refer to isolates with unresolved phylogenetic position. To view a full-size version of this figure, please visit www.cdc.gov/ncidod/eid/vol10no9/04-0197-G2.htm

Goiás and a 1984 isolate from Pará, group 1B represented sporadic human cases and samples obtained during routine surveillance in western and central regions from 1984 to 1996, group 1C consisted of isolates from sporadic cases and surveillance in eastern central states from 1971 to 1998, and group 1D represented epizootic activity from 1998 to 2001. The phylogenetic position of 12 additional isolates from Pará (10), Amapá (1), and Mato Grosso (1) was not easily resolved (Figure 2), so relationships among these strains require further sequence analysis.

The four isolates from the 1972–1973 epidemic in Goiás (group 1A, Brazil73a, 73b, 73c, and 73d) differed by 2–3 nt (0.34%–0.5%) over the length of the NS5/3′NCR fragment (595 nt). One isolate obtained 11 years later (Brazil84e) from a pool of *Haemagogus janthinomys* mosquitoes collected in far northern Pará (Faro), was nearly identical to the 1973 Goiás strains (2–3 nt difference).

Group 1B consisted of 11 isolates. These included two isolates from patients who died (Brazil84h and Brazil91b), isolates from patients in Mato Grosso do Sul (Brazil92c) and Maranhão (Brazil93a), isolates from *Haemagogus* mosquitoes in western Pará (Brazil84d) and Rondônia (Brazil96a), and four isolates obtained in 1992 from mosquito pools (Brazil92a, 92b, 92d, and 92e). Although most strains in group 1B were identified in western and central regions (over a distance as large as 3,500 km^2), one isolate identified in this cluster was from the northeast region in Barra do Corda, Maranhão (Brazil93a). Isolates collected from contemporaneous *Haemagogus* and *Sabethes* mosquito pools (Brazil92a, 92b, 92d, and 92e) were 100% identical.

Group 1C formed the largest cluster, with 22 strains diverging by 0% to 3.8% (0–27 nt, bootstrap 80%). These variants were distributed during an extended period from 1971 to 1994 throughout the northern Amazon region (Pará: Brazil87, 91a, 84c, 84g, 71, 78a, 84b, 94c, and 98a; Maranhão: Brazil80c, 82a, 93b, 94f, and 95), in central Goiás (Brazil88a, 80a, and 91c), and near the edge of the enzootic zone in Minas Gerais (Brazil89, 88b, 94d, 94b, and 94a). A pair of Minas Gerais isolates (Brazil94b and 94a) obtained from *Haemagogus* and *Sabethes* mosquito pools showed a 100% match across the length of the sequence fragment.

With one exception (Brazil98a), all isolates collected during 1998 to 2001 fell within a single cluster (group 1D) that had 0%–2.7% (0–16 nt) divergence. Although group 1D consistently clustered on neighbor-joining and parsimony trees, bootstrap support was low (53%). The distribution of samples collected during this period closely reflected the southward dispersal of epizootic activity. In 1998, a large number of YF cases occurred in northern Brazil, especially in Pará. In 1999 through 2000, a few cases were reported in Bahia and Tocantins, but the largest number were in central Goiás (19). In 2001, YF cases were

detected in the transitional zone of Minas Gerais and further east. The isolate from Tocantins collected in 2000 (Brazil2000a) was characterized by an exceptionally large number of substitutions in the 3′NCR region. A single isolate (Brazil98a) collected from a howler monkey in Afua, Pará, during the early period of the epizootic did not cluster with the other group 1D strains.

Subclade of Older Pará Strains

A group of ten isolates from Pará dating from 1954 through 1968 differed from all other Brazilian strains by 5.7% ± 1.6%. These results confirmed previous observations based on analysis of prM/E gene sequences (14), indicating 7.8% ± 2.0% divergence for this group of older Pará isolates. To date, 29 YFV strains from Pará dating from 1969 through 1999 have been examined; none of these more recent strains cluster with the earlier clade. Sequence divergence of the older Pará strains approached the threshold level used to define separate YFV genotypes (8%–9%) (20).

Alignment of previously published sequences for the structural gene region (223 codons of prM/E) (14) showed that 8 of the 10 older Pará strains had one or more mutations within the fusion peptide of the E protein (Figure 3) that is highly conserved among all flaviviruses; it is located in domain II (E98–E110) and plays a role in mediating the acid-catalyzed fusion of virions with target cell membranes (22). Three strains from 1968 (Brazil68a, 68c, and 68d) showed a C→F substitution at E105, eliminating one of the disulfide bonds that forms the structural architecture of domain II (22). Brazil68b had a D→G mutation at E98, and Brazil54 and Brazil68a shared an R→K mutation at E99. Three of the strains (Brazil55c, Brazil60, Brazil62b)

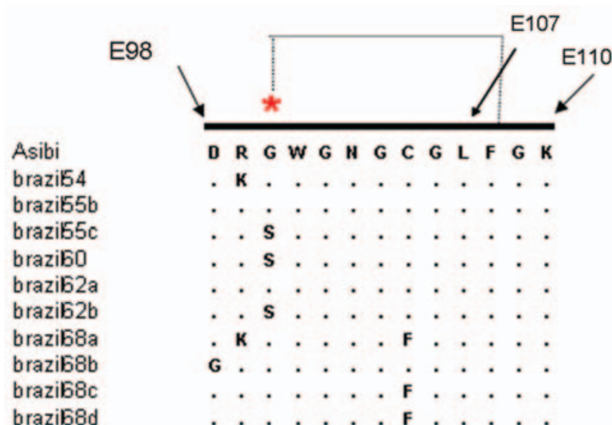

Figure 3. Sequence alignment of the fusion peptide of the envelope (E) gene of selected yellow fever virus (YFV) strains (E98–E110). The Asibi prototype strain indicates the conserved sequence present in the majority of YFV strains and other mosquitoborne flaviviruses. A salt bridge between residues Asp E98 and Lys E110 generates the "CD loop" of residues E100–E108 (21).

had a G→S substitution at E100. The functional importance of these mutations is unknown. Of the older Pará strains, only Brazil55b and Brazil62a had the consensus sequence for the fusion peptide (Figure 3). In addition, 12 non-Pará subclade strains exhibited substitutions in the fusion peptide sequence.

Preliminary phenotypic differences among three of the older Pará strains (Brazil55b, Brazil60, and Brazil68c) in the standard mouse neuroinvasiveness model (i.e., intraperitoneal injection of 8-day-old suckling mice) (23) indicated that the strains with substitutions in the fusion peptide were less lethal (average lethal dose-50 [LD_{50}] for Brazil60 and Brazil68c = 5.5 $\log_{10}$ tissue culture infective dose-50 [$TCID_{50}$]) than the strain with the consensus sequence (Brazil55b) (LD_{50} = 0.1 $\log_{10}$ $TCID_{50}$). Average survival times of the infected mice were 9.2, 11, and 8.8 days, respectively, for the three strains. All three strains achieved titers 6–7 $\log_{10}$ $TCID_{50}$/mL when grown in Vero cell culture. Brazil55b had a longer passage history than other YFV strains (4 passages in suckling mouse brain) (online Appendix); thus the altered phenotype may be partly attributed to selection during repeated mouse passage.

Additional Brazilian Isolates

Two Brazilian strains (Brazil83 from Rondônia and Brazil75 from Mato Grosso) failed to group within either of the two major subclades. Brazil83 appears to represent South American genotype II, as it matched 97.2% to the homologous NS5/3′NCR region of Peruvian YFV strains (14) and diverged from other Brazilian strains by 6.2% to 13.8% (mean 10.2%). Brazil75 was a vaccine virus, as it matched the 17DD vaccine virus by 99.8%. Sequence comparison of 17DD with Brazil75 revealed a single mutation (C→T) at position 16 of the 3′NCR (13).

Discussion

Brazil accounts for approximately 25% of all YF cases reported from South America (4). YFV activity has been reported from each of the five regions in the country (2,24). From 1950 to 2003, no single region consistently produced the highest number of YF cases; however, YFV activity in the dry northeast was rare (Figure 4). Overall, the largest number of cases was reported from Goiás (central-western) and Pará (northern) regions.

Previous studies of the genetic relationships among global variants of YFV have indicated that divergence across the length of the ≈11-kb genome is relatively uniform, and the 3′NCR contains useful markers for subtype-specific distinctions (13,25). Our data showed a single genotype of YFV circulating in Brazil (South American genotype I), with the exception of a single strain from Rondônia (South America genotype II). The Brazilian strains made up two subclades: a group of older strains

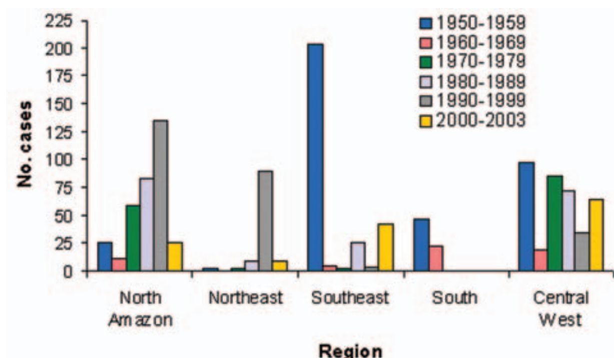

Figure 4. Yellow fever incidence in Brazil by region, 1950–2003.

from Pará from 1954 to 1968 and a larger group dating from 1969 to the present. The clear genetic distinction between strains isolated before 1968 (10 in total) and all subsequent strains suggests that the older Pará strains have been replaced by a dominant new lineage. Several members of the older Pará subclade had one or more mutations within the fusion peptide region of the E protein, including substitutions that disrupted conserved disulfide bridges (Figure 3). Although the functional importance of these substitutions is unknown, changes in the fusion peptide would affect protein folding and conformation and binding interactions with target membranes (22). Continued sampling and surveillance of YFV strains from Pará are necessary to confirm whether the variants represented in this subclade have truly become extinct or whether they are being maintained in cycles that have yet to be identified.

At least three articles in the past 50 years have reported YFV epizootics that began in northern and western Amazonian regions and then spread south into Paraná, Santa Catarina, and Rio Grande do Sul. The first was a large epidemic that swept south from Goiás in the 1930s and 1940s (26). The second was in 1963, when an epizootic started in Mato Grosso and extended eventually as far south as Missiones and Corrientes Provinces in northern Argentina by 1966 (27). In 1972 to 1973, an outbreak occurred in Goiás, and although investigations at the time suggested the epizootic was highly localized (28), cases reported from Paraguay in the following years were attributed to spread of viruses from Goiás. Virus maintenance and dispersal have presumably involved sequential infections of migrating groups of monkeys (29–32). Four isolates collected during the 1972–1973 Goiás outbreak were included in this study; these isolates (group 1A, Brazil73a–d) had nearly identical (99.6%) NS5/3′NCR sequences, and a fifth isolate with a highly similar sequence was obtained 10 years later from Faro, in northern Pará. Other than the single Faro isolate (Brazil84e), no additional descendents have been identified of those outbreak strains.

YFV transmission was particularly active during the rainy season in Maranhão in 1993 to 1994 (33). Serologic studies of rural and urban populations at the time indicated an overall attack rate of 75 per 1,000; incidence of clinical disease was 3.5 per 1,000 persons in urban areas and 4.2 per 1,000 in rural areas. Five of the six Maranhão isolates from this time were in group 1C (Figure 2), whereas one isolate from a patient with a fatal case (Brazil93a) appeared to be related to group 1B (Figure 2). In addition to the 1993–1994 outbreak in Maranhão, the subclade of group 1C strains was also associated with sporadic cases throughout 1971 to 1998 in Pará, Minas Gerais, and Goiás.

The most recent increase in epizootic YFV activity in Brazil occurred from 1998 to 2001, with cases distributed over a large region covering eight states (4). Several human cases were reported to have originated from the Chapada dos Veadeiros National Park, a tourist canyon located near Brasilia, Goiás. The proximity to major cities raised alarm, and reports at the time designated Goiás as the epicenter of the outbreak (4). The phylogenetic evidence presented here indicates that YFV activity in 1998 in Pará, in 2000 in Goiás, and in 2001 in Minas Gerais was all part of one continuous epizootic, which dispersed genetic variants from the 1D group of viruses (Figure 2).

Investigations in 2000 and 2001 indicated that YFV activity had expanded beyond the typical borders of the enzootic zone, into areas where the virus had not been reported for >100 years. The appearance of nearly identical variants across very large distances over short periods (e.g., group 1D spanning >3,000 km within 1 month, group 1B strains with isolates >2,000 km apart within 1 year) suggests that humans, rather than other primates or mosquitoes, may be partly responsible for the spread of YFV variants. Because *Haemagogus* mosquitoes are forest species and are relatively fragile, patterns of traffic and commerce would not likely lead to translocation of infected mosquitoes. From 1998 to 2001, the virus may have been transported from Pará (34) to Goiás (4) and then to Minas Gerais by asymptomatic carriers or viremic persons in the prodromal phase. The general trend in the southward movement of the virus (group 1D, Figure 2) could be interpreted to reflect the pattern of labor migration from less populated areas of the northern Amazon to cities of the southeast. However, humans are not believed to play a major role in either virus maintenance or dispersal within South America, because human contact with the forest species *Haemagogus janthinomys* is infrequent, and the length of the viremic period in infected humans is brief (3–4 days).

The identification of one strain from western Rondônia (Brazil83) with high identity to South American genotype II strains (from Peru and Bolivia) suggests that the two

South American YFV genotypes cocirculate in regions of western Brazil. The genetic divergence and distribution of YFV are similar that of Oropouche virus (an *Orthobunyavirus* transmitted by midges). Like YFV, Oropouche virus has split into two distinct genotypes in South America with separate eastern and western regions of circulation, and the only region where the two genotypes have been found to cocirculate is in western Brazil, in Rondônia (35). The ecologic correlates for these patterns remain uncertain; however, they suggest that surveys of the border regions between Brazil, Peru, and Bolivia may identify additional YFV genotypes.

One unanticipated result of this study was identifying a vaccine virus (Brazil75) from what had been presumed to be a case of natural exposure to wild-type YFV. This isolate was obtained from the blood of a patient who died in Aripuanã, Mato Grosso; this patient had been vaccinated 5 days before becoming ill and died 9 days postvaccination. Serious adverse effects resembling wild-type YF (viscerotropic disease) have only recently been reported (19,36,37). The complete genomic sequence of Brazil75 is currently being sought to address the question of vaccine reversion. Although no controlled studies have examined the safety or efficacy of YF vaccination in immunosuppressed patients, these findings underscore the importance of evaluating patient history before delivering vaccine and also suggest that other cases of vaccine-related illness may have been misdiagnosed in the past.

In summary, we describe considerable genetic variability among YFV variants circulating in Brazil and identify clusters of strains associated with epizootics in different geographic regions. Brazilian YFV strains have diverged into two subclades, one of which has become the dominant lineage in recent years. We suggest a potential role for human migration in mediating virus dispersal. Expansion of YFV outside the enzootic zone presents an ongoing risk for reintroduction of the virus to urban areas and highlights the need for continued surveillance and control.

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Genotyping, Orientalis-like *Yersinia pestis*, and Plague Pandemics

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Three pandemics have been attributed to plague in the last 1,500 years. *Yersinia pestis* caused the third, and its DNA was found in human remains from the second. The Antiqua biovar of *Y. pestis* may have caused the first pandemic; the other two biovars, Medievalis and Orientalis, may have caused the second and third pandemics, respectively. To test this hypothesis, we designed an original genotyping system based on intergenic spacer sequencing called multiple spacer typing (MST). We found that MST differentiated every biovar in a collection of 36 *Y. pestis* isolates representative of the three biovars. When MST was applied to dental pulp collected from remains of eight persons who likely died in the first and second pandemics, this system identified original sequences that matched those of *Y. pestis* Orientalis. These data indicate that *Y. pestis* caused cases of Justinian plague. The two historical plague pandemics were likely caused by Orientalis-like strains.

Yersinia pestis, a group A bioterrorism agent (1), causes plague, a reemerging zoonotic disease transmitted to humans through flea bites and typically characterized by the appearance of a tender and swollen lymph node, the bubo (2). This organism has been subdivided into three biovars on the basis of their abilities to ferment glycerol and to reduce nitrate. Based on their current geographic niche and on historical records that indicate the geographic origin of the pandemics, researchers have postulated that each biovar caused a specific pandemic (2,3). Biovar Antiqua, from East Africa, may have descended from bacteria that caused the first pandemic, whereas Medievalis, from central Asia, may have descended from the bacteria that caused the second pandemic. Bacteria linked to the third pandemic are all of the Orientalis biovar (3). In this study, we tested this hypothesis for the first time by detecting biovars in ancient human remains.

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No molecular biology–based method proved reliable and convenient for *Y. pestis* genotyping. Genome sequences of *Y. pestis* strain CO92, a Orientalis biovar, and *Y. pestis* strain KIM, a Medievalis biovar, are now available (4,5), which provides an opportunity to examine them for differences associated with the biovar and for genotyping. Genome analysis of the closely related *Rickettsia prowazekii* (6) and *R. conorii* (7) showed that intergenic spacers, which have been submitted to less evolutionary pressure than coding sequences, may be variable enough to differentiate closely related microorganisms. We, therefore, hypothesized that sequencing of several intergenic spacers would allow determination of a biovar-specific spacer pattern in *Y. pestis*. We named this method multiple spacer typing (MST). We first demonstrated that MST allowed biovar genotyping of a large collection of *Y. pestis* isolates and further applied it to the dental pulp collected from persons whose deaths are attributed to the first and second pandemics.

Methods

Bacterial Strains

Thirty-five strains representative of the three *Y. pestis* biovars (11 Antiqua isolates, 12 Medievalis isolates, and 12 Orientalis isolates) isolated from 1947 to 1996 from various host species in 13 countries are presented in Table 1. Nineteen of these isolates have been previously characterized by Achtman et al. (8). Nucleic acid was extracted as previously described (9), and species identification was confirmed for all the strains by partial sequencing of the *rpob* gene (10).

Table 1. Alleles of eight spacers in three *Yersinia pestis* biovars

Biovar	YP no. strains	Country	YP1	YP3	YP4	YP5	YP7	YP8	YP9	YP10	Isolate type
Antiqua											
	611/Japan	Japan	1	4	1	3	1	1	1	1	1
	552/Margaret	Kenya	1	3	1	1	4	1	1	1	2
	548/343	Belgium	1	3	1	1	5	1	1	1	3
	544	Congo	1	3	1	1	7	1	1	1	4
	549	Kenya	1	3	1	1	8	1	1	1	5
	542	Belgium	1	3	1	1	6	1	1	1	6
	550	Congo	1	3	1	1	9	1	1	1	7
	553	Kenya	1	3	1	1	7	1	1	1	4
	566	Kenya	1	3	1	1	6	1	1	1	6
	677	Kenya	1	3	1	1	9	1	1	1	7
	545	Kenya	1	3	1	1	7	1	1	1	4
Medievalis											
	519/PKH-4	Kurdistan	2	2	2	2	1	1	1	1	10
	616/PAR-13	Iran	2	2	2	2	1	1	1	1	8
	557/PKR292	Kurdistan	2	2	2	2	4	1	1	1	9
	564	Kurdistan	2	2	2	2	6	1	1	1	10
	565	Turkey	2	2	2	2	5	1	1	1	8
	557	Kurdistan	2	2	2	2	4	1	1	1	9
	518	Kurdistan	2	2	2	2	5	1	1	1	8
	520	Kurdistan	2	2	2	2	5	1	1	1	8
	560	Kurdistan	2	2	2	2	5	1	1	1	8
	561	Kurdistan	2	2	2	2	5	1	1	1	8
	617	Iran	2	2	2	2	5	1	1	1	8
	670	Kurdistan	2	2	2	2	5	1	1	1	8
	1594	Kurdistan	2	2	2	2	9	1	1	1	11
Orientalis											
	304/6-69	Madagascar	1	5	1	1	1	2	2	1	12
	685	Germany	1	5	1	1	2	2	2	1	13
	Hamburg10	USA	1	5	1	1	2	2	2	2	14
	CO92	USA	1	5	1	1	2	2	2	1	13
	507	Vietnam	1	1	1	1	6	2	2	1	15
	1513	Madagascar	1	5	1	1	2	2	2	1	16
	571	Brazil	1	5	1	1	4	2	2	1	17
	613	Myanmar	1	5	1	1	3	2	2	1	18
	643	Madagascar	1	5	1	1	3	2	2	1	18
	695	Germany	1	1	1	1	4	2	2	1	17
	772	Vietnam	1	5	1	1	4	2	2	1	17
	989	Vietnam	1	5	1	1	1	2	2	1	19

Spacer Sequence Database and Phylogenetic Analyses

We analyzed the complete genome sequences of *Y. pestis* strain CO92, biovar Orientalis (GenBank accession no. NC-003143) (4) and *Y. pestis* strain KIM, biovar Medievalis (GenBank accession no. NC-004088) (5), which were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (11). We used the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to determine the primer sequences specific for the genomic segments of interest (12). The primers flanked intergenic sequences of *Y. pestis* CO92 that exhibited large sequence differences with the homologous *Y. pestis* KIM strain sequences. We generated a list of *Y. pestis* CO92 intergenic sequences of 50 to 300 bp and carried out BLASTN searches to identify the homologous intergenic sequences in *Y. pestis* KIM strain by using the *Y. pestis* CO92 genes flanking the intergenic sequences as queries (13). When both genes flanking the intergenic sequence exhibited best-matches with the BLAST score >120 bits, we estimated the length of the corresponding intergenic sequence in the *Y. pestis* KIM strain. We then aligned the homologous intergenic sequences (≤300 bp) and selected eight pairs of sequences with insertion or deletion divergence between the two *Y. pestis* biovars to locate the primers (Table 2). Two microliters of DNA extracted as previously described (9) were amplified in a 50-µL mixture containing 10 pmol of each primer; 200 µmol/L (each) dATP, dCTP, dGTP, dTTP (Invitrogen, Cergy-Pontoise, France); 1.5 U *Taq* DNA polymerase (Invitrogen); and 2.5 µL of a 50-mM solution of $MgCl_2$ in 1 x Taq buffer. Each polymerase chain reaction (PCR) was performed in a T3

Table 2. Location of primers used for PCR amplification and sequencing of eight intergenic spacers in *Yersinia pestis*[a]

Spacer	Upstream gene (N) Downstream gene (N)	Primer sequence (5′ → 3′)
YP1	*ace* K (YP03724)	AATCCCTGCAAAATGGTCTG
	ace A (YP03725)	CTGATGGGAAGCAAAGGTGT
YP3	*glg* P (YP03938)	TCAGTGCATCCACACTGACA
	glg A (YP03939)	CGTATCGCCTTCACTAAGGC
YP4	gene ID 1176814 (YP03976)	TAATCCGCCGTGGAAATTAG
	gor (YP03977)	ACGATTATCTGGCAATTGGC
YP5	Gene ID 1175557 (YP02727)	GCATGCGCTGTTTGATATTG
	Gene ID 1175558 (YP02728)	TTATGACTCACGGACGATGC
YP7	*lex* A (YP00314)	GTAACGGGGACTGGATCTGA
	Gene ID 1173160 (YP00315)	ATAAACCGTGTGCTTCCACC
YP8	Gene ID 1175559 (YP02729)	ACGGAAATTGCCAGATTCA
	Gene ID 1175560 (YP02730)	GACTTGAGCTTCATTTGGCC
YP9	*mrd* F (YP02648)	GCGCTGATACGTGTTATTGG
	mrd E (YP02649)	TTGTTAATATCGCGGGTGGTA
YP10	*bio D* (YP02269)	ATGCTGAAACAATCGCAATG
	Gene ID 1175101 (YP02270)	CAATAAGGTGTACTCGCCGG

[a]Numbering according to the *Y. pestis* CO92 strain genome, GenBank accession no. NC-223143). PCR, polymerase chain reaction.

thermocycler (Biométra, Archamps, France) under the following conditions: an initial 5 min of denaturation at 95°C was followed by 39 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C. The amplification was completed by holding the reaction mixture for 5 min at 72°C to allow complete extension of PCR products. These products were purified by using the Multiscreen PCR plate (Millipore Corp., Bedford, MA), as described by the manufacturer. Sequencing reactions were carried out with a DNA sequencing kit (Big Dye Terminator Cycle Sequencing V2.0; PE Biosystem, Courtaboeuf, France), as described by the manufacturer. Sequencing products were purified and underwent electrophoresis with the 3100 Genetic Analyzer (Applied Biosystems) and aligned by using the multisequence alignment CLUSTALX version 1.8 (13).

For phylogenetic analyses, DNA sequences were aligned by using the CLUSTALW software, version 1.81 (13). Because variations among spacer sequences were only due to deletions (see below), every deletion was considered a unique molecular event that resulted in a unique mismatch, regardless of its length. For any position in the spacer sequence, identity of nucleotide was coded "1"; a mismatch was coded "0"; and a pairwise binary matrix was constructed with the MEGA 2.1 software package (14). Distance matrices were determined by using p-distance analysis and were used to infer dendrograms by the unweighted pair group method with arithmetic mean (UPGMA), maximum parsimony, and neighbor-joining, available in the MEGA 2.1 software package (14). We also used the maximum likelihood method within the PHYLIP software package (15).

Sources of Ancient DNA

The Anthropology Laboratory of Bordeaux University 1 studied a first series of 60 skeletons discovered during excavations in 1989 in Sens, France. The skeletons were buried in four adjoining mass graves, dated by radiocarbon to be from the 5th to 6th century A.D. (16). The second series came from a cemetery in Dreux, France, where nine mass graves were identified during excavations in 1990 (online Figure 1, available at http://www.cdc.gov/ ncidod/EID/vol10no9/03-0933-G1.htm). Each grave contained 2–22 skeletons. Ceramic fragments, found in the burial site, dated the graves from the 12th to the 14th century A.D. (17). Archeological data (burial patterns) and anthropologic data (absence of bone fractures, indications of sex and age of persons) supported the hypothesis that the two grave sites contained remains of persons who died during an epidemic. No historical written records for the Sens and Dreux grave sites exist, but comparisons with demographic models suggest that the graves resulted from the Justinian plague (6th–8th century) and the Black Death, respectively. The Saint-Côme and Saint-Damien site in Montpellier had been used as a church cemetery outside the city walls during the 9th to 17th centuries A.D. (18). Four graves have been dated as having been dug in the 13th and late 14th centuries, on the basis of their position on top of a 13th-century *remblai* (a small hill created by burying bodies) and the fact that they were behind a wall that dated from the second half of the 14th century. Dating of the different parts of this site was based on historical data, stratigraphy, the study of 7,059 ceramic fragments, and ^{14}C dating.

We collected 10 teeth from three skeletons in Sens, 4 teeth from three skeletons in Dreux, and 5 teeth from two

skeletons in Montpellier. The teeth were washed thoroughly with sterile phosphate-buffered saline and fractured longitudinally. Powdery remnants of dental pulp were scraped into sterile tubes for DNA extraction, as previously described (19). Seventeen teeth collected from contemporary dental patients in Marseille without any evidence of plague were used as negative control teeth ("negative teeth").

Amplification of Ancient *Y. pestis*

MST was applied to DNA extracted from 19 teeth from the remains of eight persons in one Justinian era burial and two graves from the time of the second pandemic, as described above. We instituted precautions to avoid bacteriologic and molecular contamination of ancient material (online Figure 2, available from http://www.cdc.gov/ncidod/EID/vol10no9/03-0933-G2.htm). Two successive experiments were conducted. Dental pulp was recovered in a *Y. pestis*–free building A by two operators (operators 1 and 2), who had never worked with *Y. pestis*, its DNA, or amplicons. Teeth were processed individually by cleaning and opening the tooth and recovering the dental pulp into a sterile tube, which was closed securely. Each tooth was processed separately in chronologic order from Justinian to Black Death material, and negative control teeth were processed at the end. New sterile forceps were used for every tooth. Dental pulps were then transported into another laboratory, B1, 200 m away from building A, and to building C, 600 m away from building A for DNA extraction. It was performed by operator 3, who also had never worked with *Y. pestis*. DNA extraction for each experiment was performed with new reagents, ordered directly from the distributors by operators 1 and 3, who followed the protocol for good practices for ancient DNA manipulation (20). Extracted DNA was submitted to a second laboratory in building B2/D for suicide PCRs (18), which were conducted with one negative control for every three specimens. Finally, amplicons were transported to building D for cloning and sequencing, which was carried out by operators 4 to 6, who had never worked with *Y. pestis*. PCR products were cloned in PGEM-T Easy Vector (Promega, Charbonnières, France), as described by the manufacturer. Six clones were cultivated in LB medium (USB, Cleveland, OH) overnight, and plasmid purification was performed by using the Promega system. Six clones were sequenced from every amplicon by consensus primers. The sequences were compared with sequences available in GenBank by using the BLAST software version 2.2.8 to ensure accurate species identity. The sequences were further compared by using the BLAST software version 2.2.8 with our local *Y. pestis* spacer sequence database to ensure proper biovar identity.

Results

Y. pestis Spacer Database

All the isolates were firmly identified as belonging to *Y. pestis* species on the basis of their phenotypic profile and partial analysis of 16S rRNA gene and *rpo*B gene sequences. A total of eight intergenic spacers located throughout *Y. pestis* genome (Table 1) yielded 2–9 alleles per spacer, resulting in a total of 23 molecular events potentially corresponding to >8 x 10^6 combinations (2^{23} molecular events). The 387-bp spacer YP1 exhibited a 183-bp deletion specific for the Medievalis isolates. For spacer YP3, we observed five alleles: Orientalis isolates 1513 and 695 exhibited a complete, 340-bp sequence, and the other Orientalis isolates exhibited a 16-bp deletion; Medievalis isolates featured a 48-bp deletion and a mutation G $\rightarrow$ A at position 135 of the spacer; Antiqua isolates exhibited a 32-bp deletion except for isolate 611, which had a 16-bp deletion and mutations $C_{164} \rightarrow T$, $C_{166} \rightarrow T$ and $A_{191} \rightarrow G$. Spacer YP3 thus differentiated every biovar. As for spacer YP4, Medievalis isolates were characterized by a 36-bp deletion, whereas Antiqua and Orientalis isolates exhibited a complete spacer. For spacer YP5, Medievalis isolates exhibited a full-length 292-bp spacer, whereas Antiqua and Orientalis isolates exhibited a 32-bp deletion at position 101 of the spacer. For spacer YP7, a total of nine alleles were found; Antiqua isolates were classified within seven alleles, Medievalis isolates within four alleles, and Orientalis isolates within five alleles. Alleles two and three were found only among Orientalis isolates. A full-length sequence of 330 bp was found for strain 549; the other isolates exhibited deletions. At position 126 in the spacer, strains 643 and 695 exhibited a 56-bp deletion; at position 133, strains 304, 1092, 507 and 571 exhibited a 49-bp deletion; at position 140, strains 611, 685, and 537 exhibited a 42-bp deletion; at position 142, strains 616, 548, 565, 518, 520, 560, 561, and 670 exhibited a 35-bp deletion; at position 149, strains 519, 542, 566, 564, and 1513 exhibited a 28-bp deletion; at position 155, strains 552, 613, 772, and 989 exhibited a 21-bp deletion; at position 162, strains 550, 677, and 1594 exhibited a 14-bp deletion; and at position 169 in the spacer, strains 544, 553, and 545 exhibited a 7-bp deletion. As for spacer YP8, Antiqua and Medievalis isolates exhibited a full-length 236-bp spacer, whereas Orientalis isolates had an 18-bp deletion at position 36 of the spacer. As for spacer YP9, Antiqua and Medievalis isolates exhibited a full-length 292-bp sequence; Orientalis isolates had an 18-bp deletion located at position 123 of the spacer. As for YP10 spacer, *Y. pestis* CO92 strain had a unique sequence of 369-bp; all the other isolates exhibited an 18-bp deletion located at position 177 of the spacer. Sequences herein determined

were deposited in GenBank (Appendix 1; available from http://www.cdc.gov/ncided/EID/vol10no9/03-0933_app1.htm).

Phylogenetic Analysis

Among the 35 studied *Y. pestis* strains, we identified three main phylogenetic clusters, each of which included only strains from a single biovar, i.e., Orientalis, Medievalis, or Antiqua, as observed in the unrooted dendrogram (Figure 1). The same topology was obtained when data were analyzed by parsimony, neighbor-joining, and maximum likelihood analyses (online Figure 3; available from http://www.cdc.gov/ncidod/EID/vol10no9/03-0933-G3.htm). Within the Medievalis cluster, strains 519 and 616, and strains 557 and 564 were grouped by pairs; no other subgroup was identified. Within the Orientalis cluster, three groups were identified; one group included strains 989, 613, 772, and 1513; a second group was made up of three pairs of strains, i.e., 304 and CO92, 507 and 571, and 685 and 1537; the third group diverged before the differentiation of the other two groups and contained strains 695 and 643. Within the Antiqua cluster, two groups were identified; one group included strains 553, 544, 545, 550, and 677, with the last two clustered; the second group comprised strains 552, 542, and 566; strains 548 and 549 did not group with either of these two groups but diverged before the differentiation of the other Antiqua strains. The Antiqua strain 611 exhibited a unique phylogenetic position. This strain differed from all other studied *Y. pestis* strains and appeared to have diverged before the separation of the three main clusters.

MST of Ancient Dental Pulp Specimens

In the 46 PCR experiments we performed on ancient tooth samples, we obtained 10 *Y. pestis* sequences (Figure 2); no sequences were found in the 51 PCR experiments with control teeth ($p < 10^{-4}$). The teeth from 7 of the 8 persons' remains yielded 10 specific sequences, 3 persons were positive for two molecular targets, but none of the teeth of 17 persons used as negative controls yielded specific sequences ($p < 10^{-4}$). YP1 PCR yielded an amplicon in one of six tested persons, YP8 PCR yielded an amplicon with identical sequence in six of six tested persons, and YP3 PCR yielded an amplicon in three of seven tested persons. When compared with GenBank database (Appendix 2, available from http://www.cdc.gov/ncided/EID/vol10no9/03-0933_app2.htm), theYP1 sequence obtained in skeleton 5 yielded complete similarity with the homologous region in *Y. pestis* C092 strain over 390 positions and 99% sequence similarity with the homologous region in *Y. pestis* KIM strain over 174 positions; the YP8 sequence obtained in the six persons yielded 99% sequence similarity with homologous region in *Y. pestis* C092 strain over

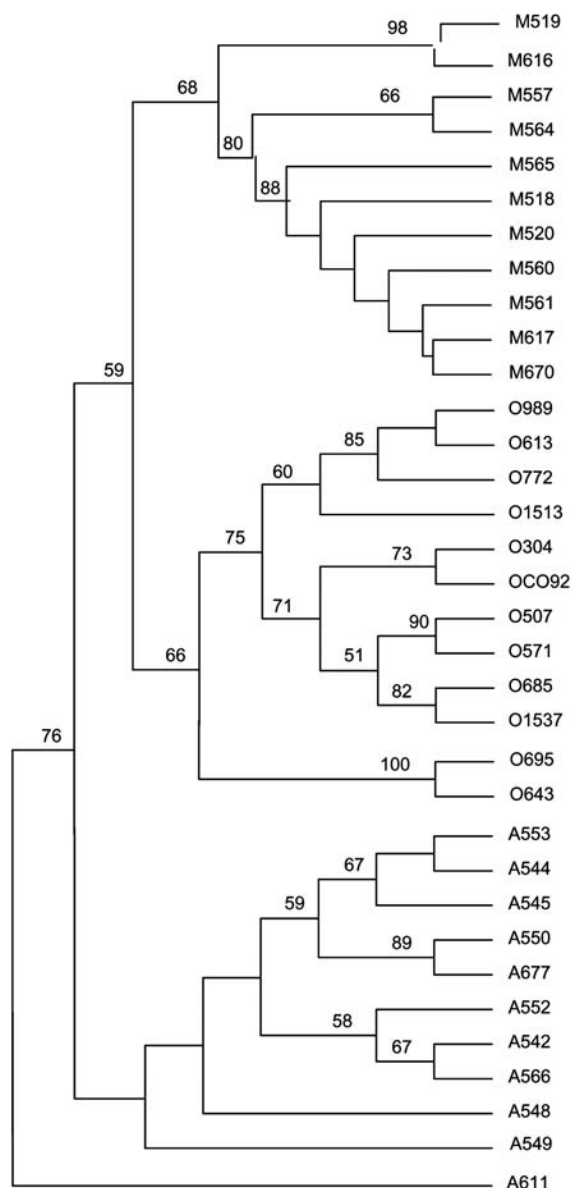

Figure 1. Unrooted tree showing the phylogenetic relationships among the 35 studied *Yersinia pestis* isolates inferred from sequence analysis of the combination of the eight intergenic spacers using the unweighted pair group method with arithmetic mean method. O, *Y. pestis* Orientalis biovar; M, *Y. pestis* Medievalis biovar; A, *Y. pestis* Antiqua biovar. Numbers refer to the isolate number as in Table 1.

178 positions and complete sequence identity with homologous region in *Y. pestis* KIM strain over 116 positions; the YP3 sequence obtained in skeletons 4 and 8 yielded complete sequence identity with that of homologous region in *Y. pestis* strain CO92 over 364 positions and 98% sequence similarity with homologous region in *Y. pestis* KIM strain over 206 positions; the YP3 sequence obtained in human remain 2 yielded a 98% similarity with homologous region

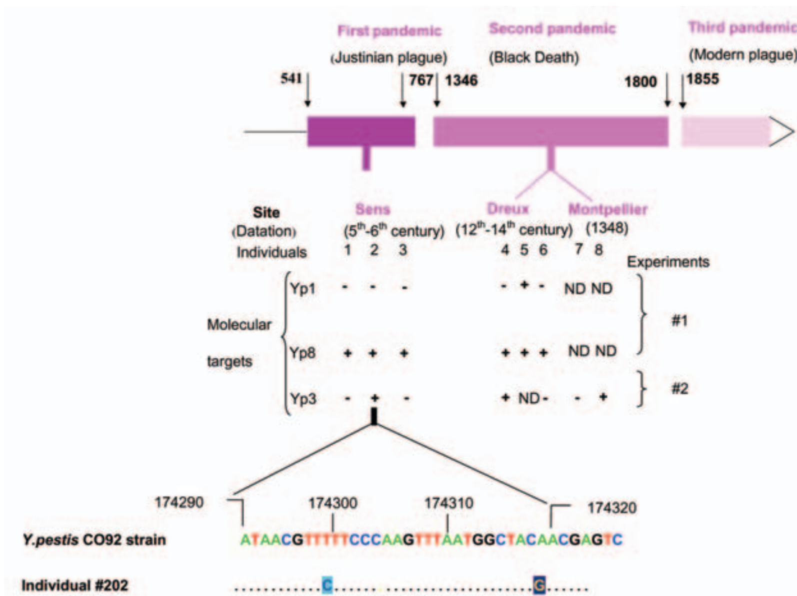

Figure 2. Molecular detection of *Yersinia pestis* was achieved in the dental pulp of remains of humans excavated from one Justinian and two Black Death mass graves in France by spacer amplification and sequencing (+, positive polymerase chain reaction [PCR] amplification and sequencing; –, absence of PCR amplification; ND, not done). Sequence analyses showed strains were of Orientalis genotype in all sets of remains; one of them exhibited two mutations numbered according to *Y. pestis* CO92 strain genome sequence (GenBank accession no. NC-003143). Negative control teeth remained negative.

in *Y. pestis* CO92 strain over 283 positions and a 95% similarity with homologous region in *Y. pestis* KIM strain over 162 positions. For each one of these 10 amplicons, further matches dropped to <90% sequence similarity over short sequences of 10 nt to 50 nt. When blasted to our local *Y. pestis* spacer sequence database, the YP1 spacer sequence obtained in human skeleton 5 was identical to that of the Orientalis and Antiqua reference sequences (Appendix 3; available from http://www.cdc.gov/ncided/EID/vol10no9/03-0933_app3.htm), whereas smaller BLAST scores were obtained for the Medievalis reference sequences. Regarding the six YP8 spacer sequences obtained in skeletons 1–6, the 12 first best scores were obtained with Orientalis reference sequences. For the YP3 spacer sequence obtained in skeletons 4 and 8, the 10 first best scores were obtained with Orientalis reference sequences. For the YP3 spacer sequence obtained in skeleton 2, the 10 first best scores were obtained with Orientalis reference sequences, and this amplicon exhibited two specific nucleotide substitutions (Figure 2; Appendix 2). These mutations were consistently obtained in six clones.

Discussion

Our data show that MST differentiates the three biovars of *Y. pestis* in a collection of 35 isolates representative of the three biovars and originating from various sources and 13 countries. This finding suggests that MST data can be extrapolated to the entire *Y. pestis* species. Pulsed-field gel electrophoresis (PFGE) has been the only other technique that allows for biovar and strain differentiation, but it requires large amounts of cultured microorganisms, and the stability of PFGE profiles in subculture has been questioned (9). Ribotyping did not classify isolates into their respective biovars (9,21,22). Specific insertion sequences (IS), including IS*100* (23), IS*285* (24,25), and IS*1541* (26,27), were used as markers in restriction fragment length polymorphism (RFLP) analyses (8,28) and in PCR-based technique (29). The last approach produced identical patterns of IS*100* distribution in Antiqua and Medievalis isolates (29). A variable-number tandem repeat technique (30,31) had a greater discrimination capacity than did ribotyping, but isolates from different areas were found to harbor identical types. Sequencing of fragments of five housekeeping genes in 36 *Y. pestis* isolates from various locations and from 12 to 13 isolates from *Y. pseudotuberculosis* and *Y. enterocolitica* did not show diversity in any *Y. pestis* housekeeping gene (8). Indeed, 19 of these 36 *Y. pestis* isolates were also included in the present work and featured 12 different MST profiles, thus demonstrating the validity of our hypothesis and the usefulness of MST for *Y. pestis* genotyping. Morever, its format is applicable to microbial analyses of ancient samples since it requires small amounts of DNA and targets small genomic fragments.

Few studies have aimed to disclose intraspecifc phylogenetic relationships of *Y. pestis* isolates. RFLP, probed with the IS*100*, indicated that, among 36 *Y. pestis* isolates, the three biovars formed distinct branches of the phylogenetic tree and that *Y. pestis* was a clone that evolved from *Y. pseudotuberculosis* 1,500–20,000 years ago (8). Isolates of biovars Antiqua and Medievalis clustered altogether apart from those belonging to biovar Orientalis. Likewise, a dendrogram constructed by the UPGMA clustering method on a PCR-based IS*100* fingerprint database clearly discriminated *Y. pestis* isolates of the Orientalis biovar that formed a homogeneous group, whereas isolates of the

Antiqua and Medievalis biovars mixed together (29). Isolates of biovar Antiqua showed a variety of fingerprinting profiles, whereas Medievalis isolates clustered with the Antiqua isolates originating from Southeast Asia, which suggests their close phylogenetic relationships. In this study, one isolate (strain Nicholisk 51) displayed a genotyping pattern typical of biovar Orientalis isolates, although this isolate was biovar Antiqua and lacked a 93-bp deletion within the glycerol-3-phosphate dehydrogenase *glpD* gene characteristic for the glycerol-negative Orientalis biovar. Our data support the view that most *Y. pestis* isolates cluster according to their biovar, but isolates of biovar Antiqua are more distantly related to other isolates than biovars Orientalis and Medievalis are. MST-based phylogenetic reconstructions unexpectedly found that one *Y. pestis* Antiqua isolate, number 611, formed a fourth branch, which suggests that *Y. pestis* may comprise four different lineages instead of the three that have been recognized so far. This unique isolate had not been included in Achtman and collaborators' study (8).

MST was applied to ancient human specimens to test the hypothesis that three biovars were responsible for the three historical pandemics. Contamination of ancient samples by modern *Y. pestis* DNA and cross-contamination were prevented in our experiments. Indeed, we carried out two independent experiments, each with new reagents, in a laboratory where *Y. pestis* had never been introduced or studied, without positive controls (17).

Both experiments produced consistent results, and negative controls were always negative. That we obtained a unique YP3 sequence further excludes the possibility of contamination, since this sequence differs from all the currently known sequences. This unique sequence was consistently found in six of six clones and thus did not result from the false incorporation of nucleotides by the DNA polymerase. In our previous work on the Black Death, we also reported a unique sequence (17). In the present study, the accurate identification of *Y. pestis* was confirmed by using two successive sequence analyses. We first blasted the sequences derived from ancient specimens against the GenBank database and observed best matches for *Y. pestis* homologous sequences, thus ensuring accurate species identification of the amplicons. We then blasted these sequences against our local *Y. pestis* spacer sequence database and found best matches for homologous sequence in Orientalis reference isolates for the three tested spacers. As our local database has 35 different reference sequences representative of the three *Y. pestis* biovars, there is no doubt regarding the identification nor the fact that Orientalis-like *Y. pestis* alone was implicated in the personal remains that we investigated. DNA of *Y. pestis* was recovered from remains of persons in one mass grave established to be of the Justinian pandemic era on the basis of radiocarbon dating (online Figure 4, available at http://www.cdc.gov/ncidod/EID/vol10no9/03-0933-G4.htm).

We found that the genotype Orientalis, which now occurs worldwide, was involved in all three pandemics. Also, we detected *Y. pestis* in additonal human remains from Black Death sites, which adds more evidence for its role in the second pandemic in southern France (four sites tested positive) (18,19). Indeed, historical descriptions were suggestive of bubonic plague in medieval southern Europe; in northern Europe, historical data indicated that the Black Death had a different epidemiologic pattern. This finding may indicate that latter outbreaks in the north were not caused by transmission of *Y. pestis* by blocked rat fleas but rather by mechanical transmission of plague bacteria by another ectoparasite that used humans as their primary hosts. Alternatively, another pathogen may have caused these outbreaks, and a search for *Y. pestis* in the dental pulps of suspected plague victims in Copenhagen (two persons' remains) and Verdun (five persons' remains) dating from the 18th century failed to show *Y. pestis* DNA (32). Further studies may elucidate the respective role of *Y. pestis* and other pathogens that may have contributed to deaths in these times.

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Rotavirus Serotype G9P[8] and Acute Gastroenteritis Outbreak in Children, Northern Australia

Carl Kirkwood,*†‡ Nada Bogdanovic-Sakran,* Graeme Barnes,*†‡ and Ruth Bishop *†‡

During 2001, an outbreak of severe acute gastroenteritis swept through Central and northern Australia and caused serious disruption to health services. We tracked and characterized the rotavirus strain implicated in the outbreak. Comparison of the electropherotypes of outbreak samples suggested that one G9P[8] strain was likely responsible for the outbreak. Samples were obtained from geographically distinct regions of Australia where the epidemic had occurred. The outbreak strains showed identical nucleotide sequences in genes encoding three rotavirus proteins, VP7, VP8, and NSP4, but they were distinct from G9P[8] strains isolated in previous years. Several of the amino acid substitutions on the VP7 and NSP4 proteins were identified in regions known to influence function and may have contributed to the emergence and increased dominance of the outbreak strains. Rotavirus serotype surveillance should continue with methods capable of identifying new and emerging types.

Rotaviruses are the major cause of severe gastroenteritis in young children worldwide. Surveillance studies and serum antibody studies indicate that all young children are likely to have had at least one rotavirus infection by the time they are 3 years of age. Worldwide, approximately 400,000–600,000 children in developing countries die of rotavirus-associated dehydration each year (1). Most deaths occur in developing countries because of delays in access to treatment. Despite low death rates in industrialized countries, good hygiene and sanitation do not appear to reduce the prevalence or prevent the spread of rotavirus.

Since 1983, vaccines to protect against clinically severe disease have been under development. The first vaccines were aimed at providing specific protection against the serotypes G1, G2, G3, and G4, which were predominant since 1973 (2). Rotavirus surveillance programs in Bangladesh (3), Brazil (4), India (5), the United States (6), and Malawi (7) show that additional G types (G5, G6, G8, G9, G10) can cause severe disease in children and are of emerging importance in some communities. Serotype G9 is recognized as the most widespread of the emerging serotypes and is now considered the fifth major G type. The serotype has been identified since 1996 as a frequent cause of severe disease in hospitalized children from many countries, including the United States, Japan, India, Bangladesh, France, Malawi, Nigeria, Australia, China, Thailand, and the United Kingdom (3,6,8–12).

Characterizing rotaviruses into serotypes is based on differences in genetic and antigenic structure of the two outer coat proteins. The rotavirus genome is made up of 11 segments of double-stranded RNA located inside the core of a triple-layered structure. The outer capsid proteins, VP4 and VP7, elicit neutralizing antibody immune responses that are both serotype-specific and cross-reactive (13). Antigenic differences in VP4 and VP7 are the basis of the classification into G (VP7 glycoprotein) and P (protease-activated VP4 protein) serotypes. To date, 8 P serotypes and 10 G serotypes have been identified in humans by cross-neutralization tests (13,14). Epidemiologic studies have shown that serotypes G1, G2, G3, and G4, associated with P1A[8] or P1B[4], have been the most common serotypes to cause severe disease in children worldwide in the 1980s and 1990s (2). Genetic and antigenic variation has been recorded within G1, G2, G3, and G4 serotypes (15). G9 strains may be more susceptible to genetic change than these other serotypes (3,16).

The emergence of G9 strains in urban Australia in 1997, together with the increasing prevalence and persistence of this serotype, has had a major effect on healthcare services in Australia (11). G9 strains were initially identified in Central Australia in 1999 in 9% of children admitted to the

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Alice Springs Hospital but appeared not to persist, since the strains were not detected in this area in 2000 (17). During May 2001, one of the largest recorded outbreaks of severe acute gastroenteritis in young aboriginal children from remote and urban areas of Central Australia resulted in 246 emergency department visits and the hospitalization of 137 children at Alice Springs Hospital (18). All specimens from hospitalized children were rotavirus positive. The epidemic spread rapidly northward, causing outbreaks of rotavirus-induced acute gastroenteritis in many communities spread over 1 million km^2 in the northern Territory and in outback areas of southwestern Queensland and West Australia from May through July 2001 (Figure 1). The National Rotavirus Strain Surveillance Program received specimens from the hospitalized patients. Serotype analysis indicated that G9 strains were responsible for the outbreak (11).

This study describes the tracking and characterizing of serotype G9P[8] strains implicated in the outbreaks in central and northern Australia in 2001 and provides evidence that the outbreaks were caused by a single strain. The results highlight the importance of continued detailed epidemiologic and virologic studies of rotavirus serotypes that cause severe gastroenteritis.

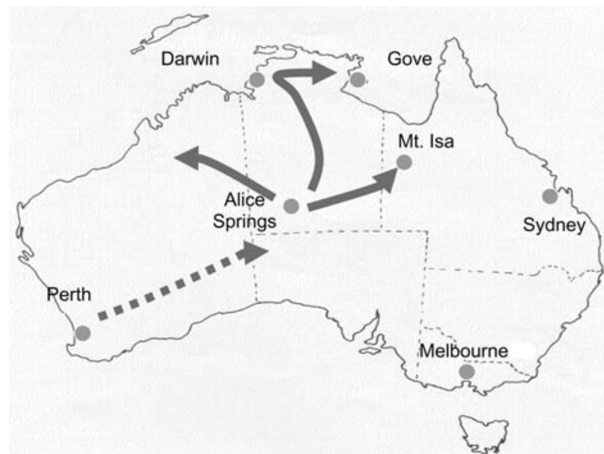

Figure 1. Map of Australia, indicating the locations where outbreaks of acute rotavirus gastroenteritis were identified during 2001. The direction of spread is indicated. Darwin-Alice Springs distance is 1,500 km.

Materials and Methods

Epidemiologic Features of Outbreaks

Alice Springs Hospital in Central Australia provides tertiary medical care for approximately 43,000 people living in a catchment area of >1 million km^2. Approximately 28,000 people live in the city and 15,000 in small remote communities that range from 12 to 800 persons (18). Emergency transport to Alice Springs Hospital is provided when necessary by the Royal Flying Doctor Service. Each year epidemics of severe rotavirus diarrhea result in hospitalization of young children, usually during the cooler months from May to August. In May 2001, "one of the largest outbreaks of rotavirus in living memory swept through Central Australia," resulting in hospitalization of 137 children with confirmed rotavirus infection. Sixty-one percent of these children were from remote regions. More than 90% of the children were identified as aboriginal. Fifty-nine percent were <12 months of age, and 96% were <4 years of age (18). A much larger number of children were less severely affected. At one stage, the Alice Springs Hospital, the only hospital serving a scattered population of 55,000 people, had 74 of its 164 beds occupied by children with gastroenteritis. Extra nurses had to be flown 1,500 km from Darwin to assist (18).

The epidemic moved northward during the next 2 months (June and July), causing an increase in the number of children admitted to hospital with acute gastroenteritis in centers such as Darwin and Gove. These towns are >1,500 km from Alice Springs. In addition, cases of acute gastroenteritis were identified in remote communities to the northeast and northwest of Alice Springs, including Mount Isa (Figure 1).

Stool Samples

During 2001, a total of 348 specimens, examined by enzyme immunosorbent assay (EIA) or heminested reverse transcription–polymerase chain reaction (RT-PCR) analysis, or both, were identified as serotype G9P[8]. Rotavirus G serotype was determined by using an in-house EIA assay that incorporates neutralizing monoclonal antibodies specific for G1, G2, G3, G4, and G9 antigens (19,20). The EIA was supplemented by RT-PCR analysis to determine G and P genotypes (21,22) The electropherotypes of all 348 G9P[8] strains were determined by polyacrylamide gel electrophoresis (PAGE) (20).

Fifteen G9P[8] rotavirus–positive specimens were selected for sequence analysis on the basis of electropherotype, location, and timing of sample collection (Table 1). Ten specimens were representative of strains from the 2001 outbreak and included six specimens from Alice Springs and the surrounding remote communities (Docker River, Hermannsberg, and Maryvale); Gove, Mount Isa, Darwin, and Perth were each represented by a single specimen. Five G9P[8] strains collected from children admitted to hospital in the cities of Alice Springs (1999), Perth (1999), Sydney (1999), and Melbourne (2000 and 2001) were used for comparison.

RT-PCR Amplification

Rotavirus dsRNA was obtained from 10% fecal extracts by using a standard phenol-chloroform extraction and a hydroxyapatite purification method (23). The

Table 1. Characterization of rotavirus strains recovered from infants in outbreak and nonoutbreak settings

Strains	Isolation location/date	Laboratory designation
Outbreak (2001)		
Ob-Perth-1	Perth 3/26/2001	3084375
Ob-AS-1	Alice Springs 5/12/2001	151572
Ob-Her-1	Hermannsberg 5/12/2001	151496
Ob-DR-1	Docker River 5/18/2001	152245
Ob-MV-1	Maryvale 5/23/2001	152433
Ob-AS-2	Alice Springs 5/25/2001	152704
Ob-AS-3	Alice Springs 5/30/2001	153004
Ob-Dar-1	Darwin 7/5/2001	6557 739
Ob-MI-1	Mt Isa 7/12/2001	6504 7767
Ob-Gv-1	Gove 8/7/2001	19522
Nonoutbreak		
MG9.06	Melbourne 9/1/2000	
Melb- G.21	Melbourne 1/3/2001	
Syd-G9.1	Sydney 6/17/1999	991680093
Perth G9.1	Perth 9/20/1999	326924
AS-G9.1	Alice Springs 9/13/1999	8705

dsRNA gene segments, encoding proteins VP4, VP7, NSP1, and NSP4, were reverse transcribed and amplified by PCR.

Full-length gene 9 was amplified with primers Beg9 and End9 (21). For gene segment 4, primers con2 and con3 were used to amplify an 887-bp region encompassing the VP8 subunit of the VP4 gene (22). Gene segment 10 was amplified with primers complementary to the 3′ end of each RNA segment (24). A 400-bp fragment of the gene segment, encoding NSP1 protein, was amplified with internal primers (25).

Sequence Analysis

PCR products were purified with gel extraction and spin column purification (Qiagen, Inc., Hilden, Germany). The nucleotide sequence of each PCR product was determined in both directions with the dideoxynucleotide chain terminator method with the BigDye sequencing kit (Perkin Elmer-Applied Biosystems, Foster City, CA) and specific oligonucleotide primers in an automated sequencer.

Sequences of each gene segment were analyzed with the Sequencher program (Gene Codes Corp., Inc., Ann Arbor, MI) and subsequently compared with other sequences by using E-CLUSTAL W and analyzed by using the DNAdist and Neighbor programs from PHYLIP software accessed through BioManager, Australian Genomic Information Service (ANGIS, University of Sydney). The statistical significance of the constructed phylogenies was analyzed with the Seqboot program to conduct bootstrap analysis, with 100 replicates (26). Phylogenetic trees were displayed with the Treeview program. The nucleotide sequences for genes encoding the VP7, VP8, NSP4, and NSP1 of the outbreak strains described in this study have been deposited in GenBank sequence databank and assigned the accession numbers AY629560–AY629562.

Northern Hybridization Analysis

Northern hybridization analysis was carried out on four Australian G9 strains isolated during 2001, two outbreak strains from Alice Springs (Ob-AS -1 and Ob-AS-3) and two nonoutbreak strains from Melbourne (MG9.06 and Melb-G9.21). Northern hybridization was performed with whole genome probes derived from virus strains F45 (G9,P1A[8], SGII, Wa genogroup) and RV-5 (G2,P1B[4], SGI, DS1 genogroup). Probes were generated by labeling cDNA, derived by reverse transcription of purified dsRNA with random hexanucleotide primers and digoxigenin (DIG)-11-dUTP. Northern hybridization was performed with 10 ng/mL of probe and performed under stringent conditions (24).

Results

Epidemiologic Features of Rotavirus Outbreak

Serotype analysis of specimens obtained from children hospitalized during the gastroenteritis epidemic showed that rotavirus serotype G9 was the predominant type identified during the Central and northern Australian 2001 outbreak (Table 2) (17). Serotype G9 strains were the most commonly identified 69% (111/161) in Alice Springs, 81% (60/74) in Darwin, and 100% (31/31 and 25/25) in Gove and Mount Isa. Serotype G9 represented 25.2% of specimens from Western Australia. However, the results from Western Australia include samples collected from two regions, one urban (Perth) and the other remote communities in the northwestern Australian outback. G9 represented 18.6% in the urban collection and 45.1% in the remote collection. All samples obtained during the epidemic outbreak (Alice Springs, Darwin, Gove, Mount Isa, and Western Australia) had an identical long electropherotype (Figure 2A), which was distinct from the patterns of

Table 2. Serotyping results from Australian centers January–December, 2001

Location	n	% of rotavirus-positive samples by serotype					
		G1	G2	G3	G4	G9	NT[a]
Western Australia							
Urban	306	67.5	0.3	0.3	–	18.6	15.1
Remote	102	34.3	1	–	1	45.1	18.9
Northern Australia (including Mt. Isa)							
Alice Springs	161	24.8	–	–	–	69	6.2
Darwin	74	5.4	–	–	1	81	12.2
Gove	31	–	–	–	–	100	–
Mt. Isa	25	–	–	–	–	100	–
Southern Australia							
Melbourne	176	48.3	4.6	0	6.8	10.2	30.1

[a]Nontypeable results include samples with mixed serotype results and samples that do not react with any of the serotyping monoclonal antibodies.

serotype G9 strains identified in other Australian locations (Melbourne, Sydney, Perth, and Alice Springs) during previous rotavirus seasons (Figure 2B).

Sequence Analysis

The gene encoding VP7 was sequenced for nine representative strains isolated from several locations during the outbreak (Ob-Perth-1, Ob-AS-1, Ob-Her-1, Ob-DR-1, Ob-Mv-1, Ob-AS-3, Ob-Dar-1, Ob-MI-1, and Ob-Gv-1). The VP7 genes were highly conserved, and all outbreak strains were identical at both the nucleotide and amino acid level. Comparison of the VP7 gene between the outbreak strains and other serotype G9 strains isolated from 1999 to 2000 in Melbourne before the outbreak (MG9.06, Melb-G9.21, and Syd-G9.1), Perth (Perth G9.1) and Alice Springs (AS-G9.1), indicated a highly conserved gene. The VP7 genes had nucleotide and amino acid homology of >99% identity. Alignment of the deduced amino acid sequences of the VP7 gene showed three conserved amino acid substitutions between the outbreak and nonoutbreak strains (Figure 3). These were at position 242 (Asn–Ser) in the antigenic F region, at position 68 (Ala–Val) in antigenic region D and at amino acid position 40 (Leu–Phe), which is outside of the major antigenic regions.

The nucleotide sequences of the VP8 subunit of the VP4 gene were determined and compared for three outbreak strains (Ob-Perth-1, Ob-AS-1, and Ob-AS-2) and two nonoutbreak strains (MG9.06, Melb-G9.21). Analysis of the predicted amino acid sequence indicated that the P[8] VP4 gene of all Australian serotype G9 strains (outbreak and nonoutbreak) had an F45-like P sublineage; 9 of the 11 amino acid positions used to classify the P sublineages were conserved with the F45-like residues (27). At position 162, all of the Australian strains possessed the Wa-lineage residue of arginine, and at position 195, a Gly was observed rather than Asp or Asn. VP8 genes of the outbreak and nonoutbreak strains differed. Comparison of the amino acid sequences of the outbreak and nonoutbreak strains showed three conserved differences at positions 21 (Glu–Lys), 91 (Ile–Val), and 249 (Val–Ile).

The complete gene segment 10 sequence, encoding the NSP4 protein, was determined for four outbreak strains (Ob-Perth-1, Ob-AS-1, Ob-AS-3, and Ob-Dar-1) and two nonoutbreak strains (MG9.06, Melb-G9.21). All of the outbreak strains had identical nucleotide and deduced amino acid sequences and at least 97% nucleotide identity and amino acid homology with the nonoutbreak strains. Alignment of the deduced amino acid sequences showed five conserved differences, at aa53 (Thr–Ala), aa76 (Val–Ile), aa141 (Thr–Ile), aa142 (Ile–Val), and aa161 (Asn–Ser) between the outbreak and nonoutbreak strains.

Northern Hybridization Analysis

Northern hybridization analysis was conducted on the Australian serotype G9 strains to investigate the genomic relationship among the gene segments from the outbreak and nonoutbreak strains (data not shown). Hybridization results showed that 10 gene segments hybridized strongly with the F45 whole genome probe, indicating that the Australian G9 strains belong to the Wa genogroup. Gene segment 5 of F45 failed to hybridize in all the outbreak and nonoutbreak strains. Partial nucleotide sequence analysis of the gene segment 5 (215-620 nt) NSP1 was conducted. A GenBank search showed that this gene had greatest identity (95%) with gene 5 from the human neonatal strain ST-3. Alignment of the deduced amino acid sequences between the outbreak strains (Ob-AS-1 and Ob-AS-3) and nonoutbreak strains (MG9.06 and Melb-G9.21) showed a highly conserved region with no conserved amino acid substitutions.

Tracking Rotavirus Outbreak G9P[8] Strain

The temporal appearance of rotavirus G9 infections in each of the geographic regions studied is illustrated in Figure 4. On the basis of these results, the suggested direction of spread of this strain is indicated in Figure 1. The rotavirus outbreak G9 strain was identified first in Perth, Western Australia, in March/April, and then in Central Australia, where the peak prevalence of rotavirus diarrhea occurred in Alice Springs in May. The outbreak strain then

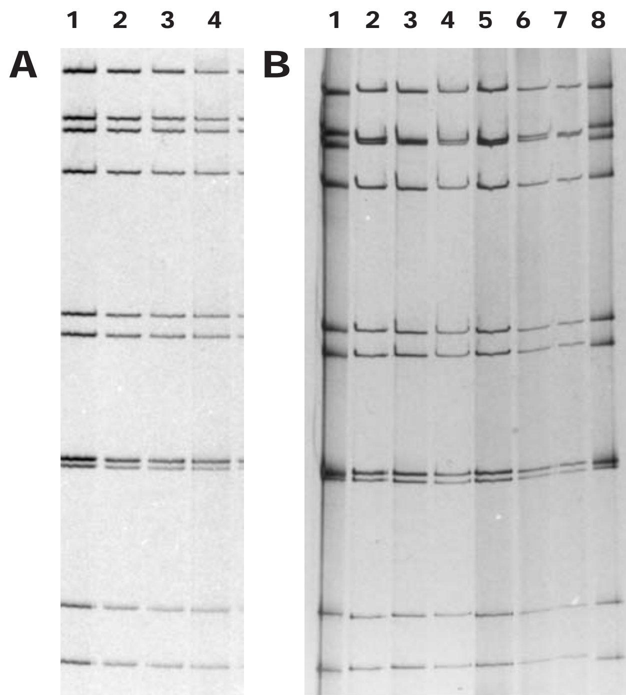

Figure 2. Electrophoretic patterns of the dsRNA of G9P[8] rotavirus strains obtained from Australian children with acute gastroenteritis during the rotavirus outbreak, 2001. Electropherotypes of four representative G9P[8] strains isolated from children during the 2001 rotavirus outbreak are illustrated in part A. Lane 1, Alice Springs (Ob-AS-1); lane 2, Darwin (Ob-Dar-1); lane 3, Gove (Ob-Gv-1); and lane 4, Mount Isa (Ob-Gv-1). B) compares the electropherotypes of G9P[8] strains isolated from children prior to the 2001 rotavirus outbreak with a strain isolated in Alice Springs during the 2001 outbreak. Lanes 1 and 8; Ob-AS-1, Lane 2; Alice Springs 1999, lane 3; Sydney 1999, lane 4; Perth 1999, lane 5; Melbourne 1999, lane 6; Melbourne 2000, lane 7; Melbourne 2001.

spread rapidly northward to Darwin in June and July. Outbreaks to the west (Docker River) and northeast (Mount Isa, Gove) of Alice Springs were also identified during June and July 2001.

Discussion

We report the genetic characterization of rotavirus G9P[8] strains isolated during the outbreak of severe rotavirus diarrhea that occurred in Central Australia during 2001. Results described in this study comprising PAGE analysis of all the outbreak strains and sequence analysis of gene segments encoding VP7, VP8, and NSP4 from representative strains illustrate that the Central Australian rotavirus outbreak was the result of the spread of a single strain of serotype G9. This strain was distinct from serotype G9 strains present in Melbourne during the same year (2001) and in previous rotavirus seasons in Alice Springs and other Australian locations, including Melbourne, Sydney, and Perth (28).

Serotype G9 is the most widespread of the emerging rotavirus serotypes. Recent epidemiologic studies suggest that this type represents a common global serotype. In Australia, serotype G9 has progressed from the initial identification of three isolates in 1997 (10) to the second most common serotype from 1999 through 2001, to becoming the most common serotype in Australia during 2002 (40% of all isolates) and 2003 (74%) (11,17). Similarly, serotype G9 was identified as the prevailing serotype in several Japanese cities from 1998 through 2000 (29).

This outbreak represented one of the largest outbreaks of rotavirus disease in Central Australian history. The outbreak, strain while appearing to have its origins in urban Perth, on the coast of Western Australia, had its major effect on remote communities in Central Australia. During May 2001, a total of 246 children with acute gastroenteritis arrived at the emergency department of the Alice Springs Hospital; 137 children were hospitalized. The outbreak stretched medical and staffing resources to capacity (18). In Alice Springs, serotype G9 represented 69% of the typeable strains. The predominance of serotype G9 increased with the northwards spread of the outbreak. The tracking of this outbreak was possible because the timing of the rotavirus activity varied by geographic location, with each community being discrete and remote from the others. This finding was highlighted in Darwin and Gove, where 81% and 100% of the isolates were identified as serotype G9, respectively. We have shown that the Central Australian outbreak strain appeared to originate from serotype G9 strains present in Perth, Western Australia, during early 2001. The Perth G9P[8] strains possessed an identical electropherotype and identical gene sequences encoding for VP7, VP8, and NSP4 proteins to strains isolated earlier during the Central Australian outbreak.

The emergence and spread of the G9 "outbreak" strain corresponded with several deduced amino acid changes in viral proteins VP8, VP7, and NSP4 compared with nonoutbreak strains from Melbourne, Sydney, Perth, and Alice Springs. These proteins have previously been shown to influence virulence of rotavirus strains (30,31). Conserved changes in the gene coding for VP7 were identified in two important antigenic regions (regions D and F). These genetic changes likely affect the function of the VP7 protein virulence. One change is adjacent to the glycosylation site Asn-X-Thr at residues 69–71, a site common to all human rotavirus strains. The substitution at position 68 (Ala–Val) may influence glycosylation and hence alter the antigenic reactivity of this virus. An effect of glycosylation on virus antigenicity and virulence has been previously postulated (30,32). Additional evidence that the removal or addition of N-linked carbohydrates can influence viral antigenicity comes from several studies using N-MAbs

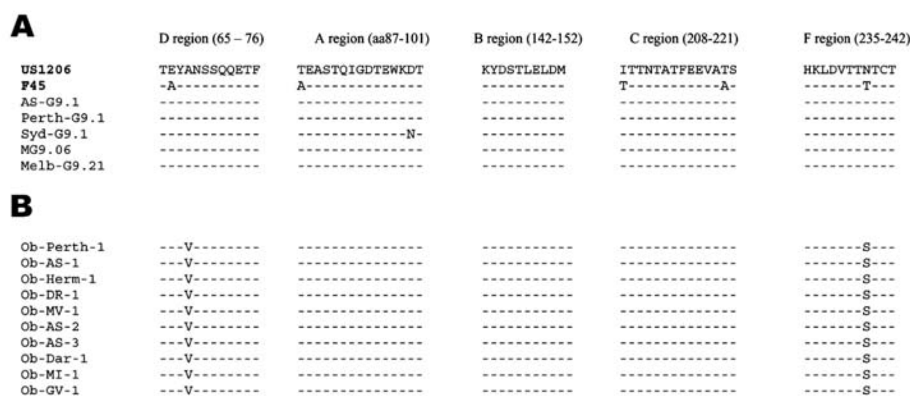

Figure 3. Deduced amino acid sequences of the VP7 antigenic regions of rotavirus G9P[8] strains. A) Outbreak strains. B) Nonoutbreak strains. The VP7 sequences of the standard G9 strain (US1206) and the Australian nonoutbreak strains were obtained from GenBank. Accession numbers are as indicated: US1206: AJ250271, Perth G9.1: AY307094, Syd-G9.1: AY307093, MG9.06: AY307085, Melb-G9.21: AY307090. The sequence of rotavirus strains F45 was obtained from Kirkwood et al. (33). All other sequences were determined in this study. A dash indicates homology with the US1206 sequence at that position.

and polyclonal antiserum (32,33). The conserved substitution identified in the antigenic F region of the VP7 protein may also be important in virulence. This antigenic region has previously been shown to contain neutralization epitopes of serotype G9 viruses, and the region was only accessible in viruses that lacked glycosylation site in this region such as serotype G9 (33). This region may represent an immunodominant region in G9 viruses. The alterations in this region identified in this study may have altered the antigenicity of the strains such that they were able to avoid immune detection. This scenario has been used to explain the reemergence of rotavirus serotypes, in particular serotype G2, and the resultant intermittent epidemics. Alterations in the antigenicity of rotavirus serotype G2 strains were conferred by amino acid substitutions in the antigenic A region of the VP7 protein. A higher incidence of infection with these strains occurred in older children in the United Kingdom from 1995 through 1999, which suggests that cross-protective antibody failed to afford protection against these serotype G2 strains (34).

NSP4 has been shown to act as an enterotoxin in mice and is involved in virus pathogenesis by acting as a receptor for double-layered particles (35). Several studies have found that NSP4 has been associated with altered virulence, by sequence comparison of symptomatic and asymptomatic strains isolated from serotypically identical human strains (30,31) or symptomatic and asymptomatic porcine strains tested in a mouse model (36), or by gene reassortment studies in a piglet model (37). Our study has identified changes in important regions of this protein of the outbreak strain. Specifically, changes have been identified in a region of NSP4 critical for VP4 binding (aa112–148) and in a region associated with membrane destabilization (aa48–91) (38). These changes may affect virus stability. NSP4 has been shown to elicit an immune response in humans (39). However, the influence of these changes identified on immune recognition is unknown, since the antigenic regions are uncharacterized.

The electropherotype of the G9P[8] outbreak strains differed in the several respects from G9P[8] strains identified elsewhere in Australia from 1999 to 2001. Differences were most apparent in the mobility of gene segments 2 and 3. As yet, no evidence shows that genes 2 and 3 (coding for VP2 and VP3, respectively) are involved in virulence (37).

Northern hybridization analysis of both outbreak and nonoutbreak serotype G9 strains had a similar hybridization pattern. Ten of 11 segments hybridized to a Wa-like probe, which indicated that these strains all belong to the human Wa genogroup. Only gene segment 5, which encodes the NSP1 protein, failed to hybridize. The gene 5 showed greatest identity to gene 5 of the P[6] strains ST3 and M37, rather than P[8] strains. Several studies have shown that the G9 VP7 protein is capable of associating with both VP4 proteins P[6] and P[8], in addition to VP6 proteins from subgroup I and II (3,16,40). A previous study

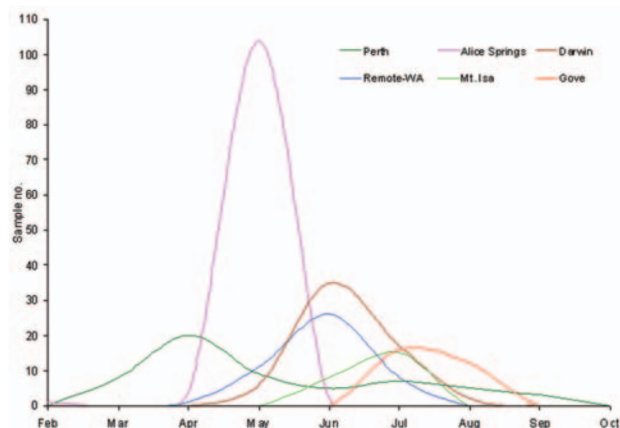

Figure 4. Temporal appearance of rotavirus G9P[8] strains isolated from children admitted to hospital with acute gastroenteritis during 2001. The monthly appearance of G9P[8] strains for each of the collaborating laboratories is indicated. The Western Australia results are divided in urban location (Perth) and remote outback locations (remote-WA).

has associated gene 5 with pathogenicity in the mouse model (41). However, alterations in the gene 5 segment cannot explain the emergence of this strain in Central Australia since limited sequence analysis failed to identify any genetic difference between the outbreak and nonoutbreak G9 strains. The results from this study further highlight the ability of serotype G9 strains to undergo reassortment and extend this observation to include gene segments that encode non-structural proteins.

Rotavirus surveillance programs using molecular assays have shown that most cases of acute gastroenteritis are associated with the globally common serotypes, G1–G4. However, the emergence of novel or rare serotypes, including the identification of serotypes G5, G6, G8, and G10, in children in many settings worldwide, has highlighted a much greater strain diversity than previously reported. The diversity of rotavirus strains has arisen because of the strains ability to undergo genetic evolution. A number of different mechanisms exist by which rotavirus strains can evolve, ranging from reassortment of single or multiple gene segments during mixed infections by strains of human-human origin or human-animal origin to generation of single point mutations in immunologically important genes. In particular, serotype G9 strains appear to have an enhanced capacity to reassort. Epidemiologic studies have identified G9 strains in combination with several different VP4 genogroups (P[4], P[6], P[8], and P[11]) and with both VP6 subgroup antigens (3,9,12,24,28,40). These mechanisms provide rotavirus with a unique capacity to rapidly evolve, and produce strains that have the potential to be epidemiologically important. Protection from rotavirus disease relies on production of heterotypic immune responses after primary infection. However, novel strains may avoid stimulating preexisting immunity produced from previous rotavirus infections because of the unique nature of the outer capsid proteins. Outbreaks of acute gastroenteritis associated with an unusual serotype G2 strain and serotype G9 are two recent examples from Central Australia (42). Therefore, the diversity of rotavirus serotypes has important implications for vaccine development, especially if strains that are not targeted by current vaccine candidates continue to emerge as common types either globally or regionally.

National surveillance data since 2001 highlights the continued emergence of serotype G9 as the most prevalent serotype nationally, which results in the replacement of serotype G1 as the dominant strain for the first time since Australian rotavirus surveillance began in 1993. This study, which shows that a single rotavirus serotype G9 strain was responsible for a large epidemic of severe gastroenteritis in Central Australia, emphasizes that sequence alterations on viral proteins may be implicated in virus virulence. Continued surveillance of rotavirus serotypes, which includes the capacity to identify new and emerging serotypes, is important for successfully developing and implementing vaccines.

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Foodborne Botulism in the Republic of Georgia

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Foodborne botulism is a potentially fatal, paralytic illness that can cause large outbreaks. A possible increase in botulism incidence during 2001 in the Republic of Georgia prompted this study. We reviewed surveillance data and abstracted records of patients with botulism who were hospitalized from 1980 to 2002. During this period, 879 botulism cases were detected. The median annual incidence increased from 0.3 per 100,000 during 1980 to 1990 to 0.9 per 100,000 during 1991 to 2002. For 706 botulism patients hospitalized from 1980 to 2002, 80% of their cases were attributed to home-preserved vegetables. Surveillance evaluation verified that botulism incidence varied greatly by region. Georgia has the highest nationally reported rate of foodborne botulism in the world. A strategy addressing individual behaviors in the home is needed to improve food safety; developing this strategy requires a deeper understanding of why botulism has increased and varies by region.

Botulism is a severe, paralytic illness caused by toxins of the spore-forming, gram-positive rod *Clostridium botulinum*. Illness is characterized by cranial nerve dysfunction and symmetric descending flaccid paralysis, which may result in death from respiratory failure (1). Foodborne botulism, the most common form, is caused by eating food containing preformed botulinum toxin. Because *C. botulinum* is ubiquitous in the environment, spores routinely contaminate food and survive standard cooking practices that do not exceed 100°C. *C. botulinum* cells produce botulinum toxin only under unique conditions: an anaerobic environment, nonacidic pH, low salinity, and high water activity (a critical factor influencing shelf-life) (2). Home-preserved foods often attain these conditions and, therefore, present a high risk for botulism when spores survive the preservation process.

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Though uncommon, botulism is a public health problem for a number of reasons (3). First, one contaminated food product can rapidly make a large number of people critically ill; even a single case represents a public health emergency. Second, botulism is highly preventable with proper techniques for preserving and preparing food. And third, the potential for intentional botulinum toxin release into food, water, or air obligates public health officials to gather more data about botulism for bioterrorism preparedness (4).

In 2001, public health officials in the Republic of Georgia (est. 2002 population 4.4 million persons) became concerned about a possible increase in the incidence of botulism. A small mountainous country in the South Caucasus bordering the Black Sea, Georgia gained independence in 1991. Although once a prosperous Soviet republic, Georgia now has a per capita annual income of US$591, making it one of the poorest countries in the world (5). Its public health system, moreover, has been challenged by massive outbreaks of vaccine-preventable diseases, declining sanitation, and an inability to fund its healthcare system (6–8).

At the time of this study, it was not known whether the reported increase in botulism incidence was real and what the possible causes were. To assess the magnitude of botulism in Georgia, we reviewed existing surveillance data and performed case finding and active surveillance in hospitals and public health offices. To further characterize the epidemiology of botulism, we abstracted records of botulism patients hospitalized from 1980 to 2002.

Methods

National Public Health Surveillance

Georgia's National Center for Disease Control (NCDC) conducts passive surveillance for botulism. Physicians are required by law to report all suspected cases of botulism to

local epidemiologists who, in turn, are required to report to region-level epidemiologists and to NCDC. Administratively, Georgia is divided into 10 regions, 2 major cities, and 2 autonomous republics. The two autonomous republics, South Ossetia and Abkhazia, are not under public health surveillance.

Medical Records Review

We visited hospitals in cities and regions of Georgia that reported at least one botulism case from 1980 to 2002. At each hospital, medical records were sorted by discharge diagnosis. A patient was considered to have botulism if medical records indicated that this was the final diagnosis. For each patient, a trained epidemiologist completed a standardized data abstraction form that included patient demographics, illness history, and clinical characteristics. A random sample of patient records (13%) was audited by a staff physician at the Tbilisi Infectious Pathology Center, which supplied most of the patients for this study, to confirm the accuracy of abstracted data; all paper abstraction forms and electronic records were compared to assess accuracy of data entry.

Outbreaks were not documented separately in national surveillance. To account for clustering of cases from common food sources, we defined an outbreak as two or more patients who were documented in the medical chart as being part of an outbreak; who had hospital admission dates no more than 3 days apart; and who had identical suspect food sources, town of residence, and hospital of admission. A botulism event was defined as an outbreak or as a sporadic case (i.e., an individual case not associated with other cases). When performed, diagnostic testing on food or human specimens was conducted at NCDC by using the standard mouse bioassay for detecting botulinum toxin (9).

Surveillance System Evaluation

Because our analysis demonstrated marked geographic variation in incidence, we performed retrospective case finding and active surveillance for botulism in two parts of Georgia that reported no cases from 1980 to 2001: the city of Poti, estimated 2002 population 46,000, and the region of Samegrelo, estimated 2002 population 405,500. For retrospective case finding in these two parts of Georgia, we interviewed local epidemiologists to identify botulism cases that were not reported to NCDC from 1996 to 2002. We also interviewed physicians working at the main outpatient clinics and on inpatient infectious diseases, critical care, and neurology wards. At hospitals, we reviewed medical records to identify suspected botulism cases among patients diagnosed with neurologic syndromes from 1996 to 2002. We also reviewed pharmacy records at these hospitals to determine whether botulinum antitoxin, which is widely available, was ever administered to a patient. For active surveillance, we telephoned epidemiologists and hospital-based physicians every month from April through December 2002 to ascertain whether any new cases of botulism occurred in these two parts of Georgia.

Home Visits

To learn more about practices in the home, we visited 14 homes in different regions of Georgia. We interviewed families about food preservation practices. We purchased tomatoes, peppers, and cucumbers at local markets, and asked persons who routinely preserve such vegetables to demonstrate this process for us.

Ethics Review

The studies reported in this manuscript were reviewed by the human subjects committees at the Centers for Disease Control and Prevention (Atlanta, Georgia, USA) and at NCDC (Tbilisi, Georgia).

Results

National Surveillance Data

From 1980 to 2002, we found 879 cases of botulism reported in Georgia. In 2002, a total of 39 cases were ascertained for an incidence of 0.9 per 100,000 persons (Figure 1). The median annual incidence increased from 0.3 per 100,000 persons (median case count 15), during 1980 to 1990, to 0.9 per 100,000 persons (median case count 41), during 1991 to 2002. The incidence was highest in 1994 (3.6 per 100,000 persons) when 173 persons became ill at a wedding from eating contaminated fish. Fifty-eight deaths were attributed to botulism, for an average death rate of 7%. The case-fatality rate ranged from 0% (several years) to 18% (in 1981). The median case-fatality rate increased from 3% (1980–1990) to 7% (1991–2002).

Medical Records Review

We identified medical records for 706 patients with a diagnosis of botulism from eight hospitals in five regions of Georgia. Though patients resided in eight regions of Georgia, 90% of patients were initially hospitalized at or transferred to one Tbilisi hospital, which serves as the national referral hospital for botulism. Because national surveillance data consisted of only summary statistics and lacked personal identifiers, we could not determine whether the 706 patients identified through hospitals represent a subset of the total reported in surveillance (879) or all hospitalized, diagnosed botulism case-patients in Georgia.

The median age of patients was 34 years (range 1–90). Three hundred fifty-five (50%) were female. Patient eth-

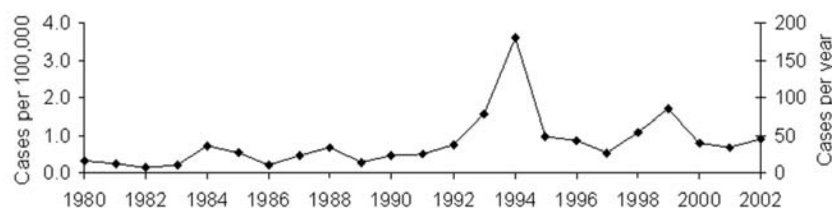

Figure 1. Number of botulism cases and cases per 100,000 persons in Georgia, 1980–2002. Data are derived from routine, passive national surveillance. Data are presented as one trend line because the incidence and absolute case count trend lines are indistinguishable.

nicities included Georgian (73%), Azerian (11%), Armenian (10%), and Russian (5%). The median age, sex, and ethnicity of patients was similar to that of the general population, except for the proportion of Azerian patients, which was almost twice as large as the proportion of Azerians living in Georgia in 1989 and 2002 (10). Fifty-four (8%) patients died during hospitalization.

We identified 329 botulism events: 154 outbreaks involving 531 persons (median 3 persons per outbreak; range 2–83) and 175 sporadic cases. Medical records were available for 83 of the reported 173 patients from the 1994 outbreak. The eastern part of the country, particularly the area surrounding Tbilisi, had the highest incidence rate (Figure 2). No botulism events were identified in three areas of the country: the city of Poti and the regions of Samagrelo and Racha-Lechkhumi.

All botulism events were suspected or confirmed to be foodborne. The most commonly implicated food was home-preserved vegetables, accounting for 261 (80%) events. Other implicated foods included smoked fish (12%) and smoked meat (2%). Among events involving home-preserved vegetables, the most commonly implicated vegetables were tomatoes (15%), peppers (15%), celery leaves (13%), eggplant (13%), and combinations of vegetables (13%). The proportion of cases attributed to vegetables did not vary significantly across years or regions. Home-preserved vegetables were implicated more frequently among patients of non-Georgian ethnicity (83%) than among patients of Georgian ethnicity (64%) ($p < 0.01$). Botulism events varied markedly by season; most occurred in the winter (55%) or spring (23%), compared to summer (10%) or fall (12%). The proportion of events attributable to home-preserved vegetables was almost two times greater in the winter (86%) than in the summer (44%) ($p < 0.01$).

A specimen from either a patient or food was collected in 116 (35%) botulism events. Of events tested, 20 (17%), involving 75 persons, had botulinum toxin detected. Among these 20 events, type B toxin was identified in at least one specimen from 17 events (85%) (3 from blood, 13 from food, 1 from vomitus), type E in 2 events (10%) (1 from blood, 1 from food), and type A in 1 event (5%) (food). Home-preserved vegetables were implicated in all 17 events in which toxin type B was identified. Smoked fish was implicated in the one toxin type A event and smoked fish and home-preserved tomatoes in the two toxin type E events.

Surveillance System Evaluation

Retrospective case finding in the city of Poti and region of Samegrelo, both of which previously reported no cases, identified three unreported, possible cases of botulism from 1996 through 2002: one involved incorrect reporting of the site of residence, the second involved failure of a physician to report, and the third involved failure of an epidemiologist to report. In the last two cases, neither patient reported eating high-risk food, neither was treated with antitoxin, and neither was given a final diagnosis of botulism by the treating physician. Review of antitoxin distribution records did not identify any patients treated with antitoxin from 1996 to 2002. Active surveillance for botulism cases did not identify any new cases from April to December 2002 in Poti and Samegrelo.

Home Visits

We observed that the home preservation process varied between homes, but the sequence of events was common. We also found that many families inadequately sterilize equipment, conserve vegetables uncooked, fail to use pressure cookers, store vegetables for months to years, eat preserved vegetables uncooked, and, out of necessity, occasionally eat food that looks and smells spoiled. Salt, sugar, vinegar, and spices are frequently added but usually in small amounts.

Figure 2. Cumulative incidence of botulism events by region of residence, per 100,000 persons, in Georgia, 1980–2002. Outbreaks and sporadic cases are counted as one event. Data are derived from review of medical records at hospitals. For comparison, the cumulative incidence rate of botulism events for the country was 6.7 per 100,000. Asterick indicates autonomous regions not under government control.

Discussion

The incidence of botulism in Georgia has been high since 1980, has increased threefold since Georgia gained independence in 1991, and varies considerably across the country. Home-preserved vegetables and smoked fish are the most commonly recognized sources of botulism.

Georgia has the highest nationally reported rate of foodborne botulism in the world. Few countries report botulism incidence. Of those that do, the highest reported rate outside of Georgia is from Russia, which reported 501 cases in 1998, an incidence of 0.3 per 100,000 (11). In 2001, the incidence in the United States was 0.01 per 100,000, although the rate in the state of Alaska was 1.6 per 100,000 (12). In the European Union, rates are all <0.1 per 100,000 (13).

Georgia's surveillance system works surprisingly well. Our evaluation in two low-incidence areas of Georgia leads us to believe that the geographic variation in incidence found through medical record review cannot be entirely explained as an artifact of surveillance. Using the annual incidence rate of 0.9, we would expect approximately four cases per year in Poti and Samegrelo. We did find three possible cases, but two were never given a final diagnosis of botulism. No cases were later identified through active surveillance. The incidence of disease in those areas, as well as across Georgia, may be underestimated because patients with mild symptoms fail to seek care, or patients with severe illness die before receiving medical attention. Economic collapse in the 1990s led the Georgian government to privatize the previously state-run healthcare system. Consequently, many persons cannot afford the cost of physician visits or hospital stays, and many Georgians may be unaware that botulism is one of the few medical conditions treated at public expense (14).

Our study has several limitations. Our evaluation of the surveillance system was limited by the use of unstructured interviews of physicians and epidemiologists. Similarly, we were unable to quantify the sensitivity of surveillance because we could not identify nonhospitalized botulism patients, and we did not have patient identifiers to link surveillance data to hospital medical records. Our medical records review included few laboratory-confirmed cases, but a separate analysis of the clinical features of botulism patients leads us to believe that misclassification was unlikely (15). Residence was obtained from the medical charts, but we do not think this is a source of bias. Many patients from outside Tbilisi sought care at facilities close to their residence and had records indicating where they were first evaluated. The geographic differences in incidence were also reflected in reporting by local public health agencies.

Even though presumably acidic vegetables (e.g., tomatoes) are frequently used, the preservation process in Georgia remains dangerous. In the absence of sterilization and pressure cooking, *C. botulinum* spores can survive and will elaborate toxin in solutions that are relatively neutral with low salt and sugar content. Failure to heat food before eating increases the risk further (1). Households probably conserve vegetables because of the absence of inexpensive, readily available, industrially preserved food, particularly in the winter, when fresh vegetables are prohibitively expensive. Dependence on home-preserved vegetables is likely to have increased since 1991 because of economic collapse, including the closure of virtually all commercial canning factories in Georgia (16). Other factors may include cultural preferences and inadequate awareness of attendant risks. Changing practices will be difficult because the intervention needs to be home-based and must account for the various forces that compel persons to eat improperly preserved food.

Why the rate of botulism has increased dramatically remains unclear. Poverty likely drives more persons to conserve food; lack of reliable energy sources, clean water, and cooking supplies makes food preservation practices riskier; and food shortage compels persons to rely on preserved food for a larger proportion of their diet. Poland historically reported high rates of botulism, but as economic conditions and food production improved in the 1990s, the incidence of botulism declined dramatically from 0.9 per 100,000 in 1990 to 0.2 per 100,000 in 1998 (17).

Wide geographic differences in incidence make us suspect that some parts of the country do not conserve food as frequently or may have developed safer techniques. These regional differences may hold the key to a successful intervention. Definitive interventions, such as developing a commercial canning industry or distributing pressure cookers, are impractical at this time because Georgia's economic infrastructure has collapsed. Instead, we may be able to prevent botulism by identifying culinary, cultural, and social factors that keep the incidence low in some parts of the country and translating those findings into a public health message for the high-incidence areas. For now, Georgia's public health message is that persons should thoroughly heat home-conserved vegetables and that medical care for botulism is free. An effective, inexpensive, culturally appropriate intervention is needed to improve food safety in Georgia.

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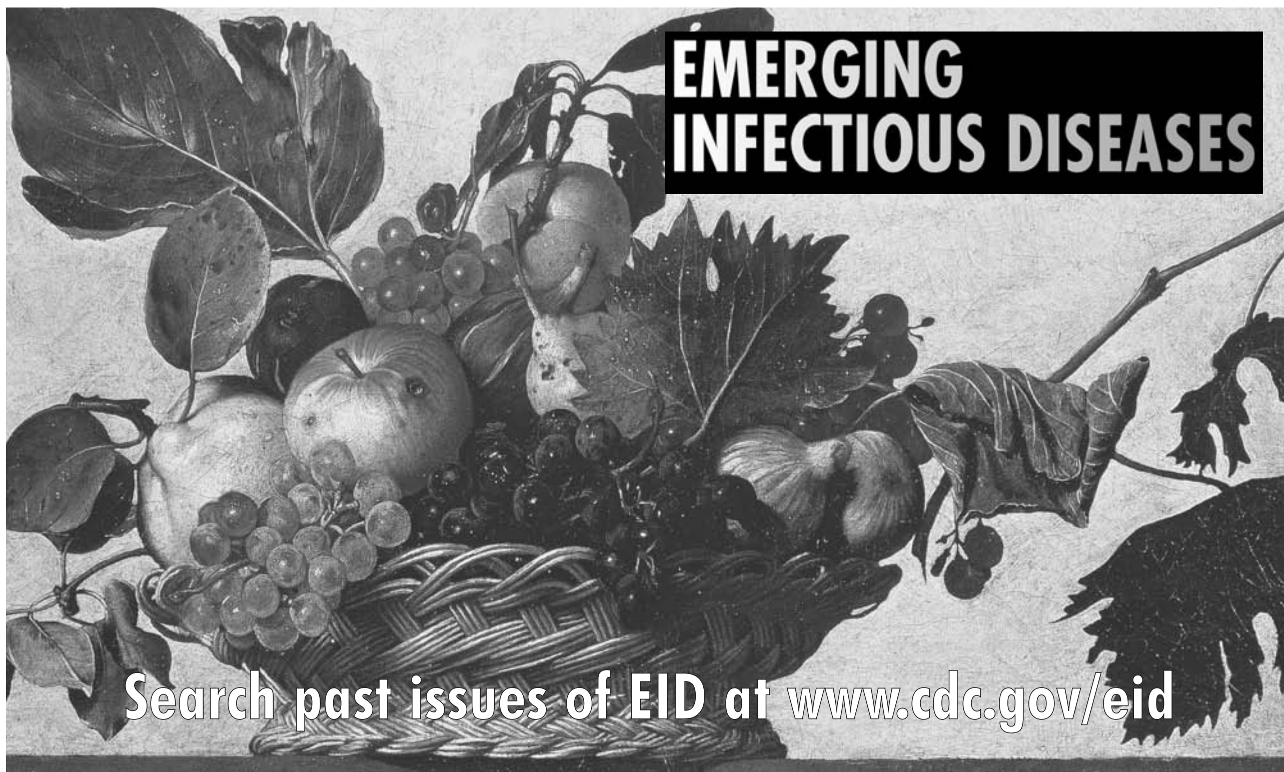

Foodborne Botulism in the United States, 1990–2000

Jeremy Sobel,* Nicole Tucker,* Alana Sulka,* Joseph McLaughlin,*†[1] and Susan Maslanka*

Foodborne botulism, a potentially lethal neuroparalytic disease, is caused by ingesting preformed *Clostridium botulinum* neurotoxin. We reviewed surveillance data and reports from 1990 to 2000. Of 263 cases from 160 foodborne botulism events (episode of one or more related cases) in the United States, 103 (39%) cases and 58 events occurred in Alaska. Patients' median age was 48 years; 154 (59%) were female; the case-fatality rate was 4%. The median number of cases per event was 1 (range 1–17). Toxin type A caused 51% of all cases; toxin type E caused 90% of Alaska cases. A particular food was implicated in 126 (79%) events. In the lower 49 states, a noncommercial food item was implicated in 70 (91%) events, most commonly home-canned vegetables (44%). Two restaurant-associated outbreaks affected 25 persons. All Alaska cases were attributable to traditional Alaska Native foods. Botulism prevention efforts should be focused on those who preserve food at home, Alaska Natives, and restaurant workers.

Botulism is a paralytic illness caused by neurotoxins of the anaerobic, spore-forming bacterium, *Clostridium botulinum*, and rarely, by botulinum toxin–producing strains of *C. baratii* and *C. butyricum*. Seven immunologically distinct toxins exist, designated A through G. Types A, B, and E cause most human cases; type F cases have been reported rarely. Botulinum neurotoxins induce blockage of voluntary motor and autonomic cholinergic neuromuscular junctions, which prevents motor fiber stimulation. Clinical illness is characterized by cranial nerve palsies, followed by descending flaccid muscle paralysis, which can involve the muscles of respiration. Although ptosis and dysarthria may be mistaken for signs of encephalopathy, patients are fully alert, and the results of a sensory examination are normal. Recovery often takes weeks to months (1). The mainstays of therapy are meticulous intensive care unit support, with mechanical ventilation if needed, and administration of equine antitoxin (2). Timely antitoxin administration may arrest the progression of paralysis and decrease the duration of illness (2).

Foodborne botulism is a rare illness caused by eating foods contaminated with botulinum toxin. Spores of *C. botulinum* are ubiquitous in the environment (3), but growth and elaboration of toxin occur only under particular conditions that include an anaerobic, low-salt, low-acid environment. Bacterial growth is inhibited by refrigeration below 4°C, heating above 121°C, high water activity,or acidity (pH <4.5) (4). Toxin is destroyed by heating to 85°C for at least 5 minutes, and spores are inactivated by heating to 121°C under pressure of 15–20 lb/in^2 for at least 20 minutes (5).

The canning and fermentation of foods are particularly conducive to creating anaerobic conditions that allow *C. botulinum* spores to germinate. Botulism was first described in consumers of sausages in Europe in the 18th century, and commercially canned foods caused outbreaks in the 19th and early 20th centuries before standard methods for inactivating *C. botulinum* spores in cans were perfected (6). Early in the 20th century, the proportion of botulism outbreaks caused by contaminated, commercially produced foods declined; however, improperly made home-canned foods have long constituted a major source of botulism in the continental United States (1,7). Since the 1970s, restaurant-associated botulism outbreaks have accounted for a large proportion of U.S. cases (8). Traditional Alaska Native foods, especially fermented foods like fish and fish eggs, seal, beaver, and whale, also pose a risk and account for the high incidence of botulism in Alaska (9). These foods, prepared by allowing the products to ferment at ambient temperatures, are often eaten without cooking.

Because contamination of a widely distributed food product could affect large numbers of persons, intensive

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surveillance is maintained for botulism cases in the United States, and every case is treated as a public health emergency. The Centers for Disease Control and Prevention (CDC) is the only source of therapeutic antitoxin, which is stocked in locations around the country for rapid release. CDC maintains a 24-hour clinical consultation and emergency antitoxin release service, and state health departments conduct epidemiologic investigations of suspected cases (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/botulism.pdf) (1). Clinicians who suspect that they are treating a case of botulism should immediately contact their state health department's emergency telephone number. Botulinum toxin has been developed as a biologic weapon by various countries and terrorist groups and could be disseminated by deliberate contamination of foods or aerosolization (10). This situation adds urgency to early recognition and reporting of botulism cases. We review surveillance data on cases and outbreaks of botulism in the United States from 1990 to 2000.

Methods

Suspected and confirmed botulism cases are reported to CDC by state health departments. Surveillance data, published and unpublished case reports, and outbreak reports were reviewed. A case of foodborne botulism was defined as a compatible illness in a person whose serum, stool, or gastric secretions tested positive for botulinum toxin; a compatible clinical illness in a person who ate food that tested positive for botulinum toxin; a compatible illness in a person whose stool was culture-positive for *C. botulinum*; a compatible illness in a person who ate food that was also eaten by a person with a laboratory-confirmed case; or a designation of the illness as foodborne botulism by the reporting state health department. An outbreak was defined as two or more cases of botulism caused by consuming a common source–contaminated food. An event was defined as the occurrence of a sporadic case or an outbreak of botulism. A food was considered the source of an outbreak if it tested positive for botulinum toxin and was eaten by a case-patient, or if an epidemiologic investigation linked a food to a botulism event, if the food was not tested. States other than Alaska were designated as the "contiguous states and Hawaii." Limited clinical data were collected at the time clinicians sought botulinum antitoxin from state health departments and CDC, and changes in data collection practices occurred during the period reported. Toxin detection and culture for *C. botulinum* were performed at CDC and 19 state and municipal public health laboratories according to standard methods (5). Limited clinical descriptors and outcome data were collected. Statistical analysis was performed using SAS version 8.2. (SAS, Cary, NC).

Results

United States

From 1990 to 2000, 160 foodborne botulism events affected 263 people in the United States, an annual incidence of 0.1 per million. No discernable trend was evident in the overall or toxin type–specific annual case counts (Figure). The median number of cases per year was 23 (range 17–43), the median number of events per year was 14 (range 9–24), and the median number of cases per event was 1 (range 1–17). The highest incidence rates were in Alaska, Idaho, and Washington (Table 1). One hundred and thirty-one cases (50%) were caused by toxin type A, 27 (10%) by toxin type B, and 97 (37%) by toxin type E (Table 2). Among case-patients for whom data were available, 67 (26%) persons were intubated. Forty-one (84%) persons with type A intoxication were intubated, compared with 6 (33%) persons with type B and 17 (33%) of those with type E. For case-patients with available data, 7 persons (5%) with type A intoxication died, compared with 1 (4%) with type B and 3 (3%) with type E.

Contiguous States and Hawaii

Events

In the contiguous states and Hawaii, 102 foodborne botulism events occurred, which affected 160 persons; a median of 9 events (range 4–13) and 14 cases (range 4–30) took place per year. The median number of cases per event was 1 (range 1–17). The median age of case-patients in the contiguous states was 50 years (range 4–88 years), and 83 (52%) were female. The overall case-fatality rate was 5%. No clear seasonal pattern was observed.

A food was implicated by laboratory detection of toxin or epidemiologic investigation without laboratory confirmation in 77 (76%) events (Table 3). Of these events, 68

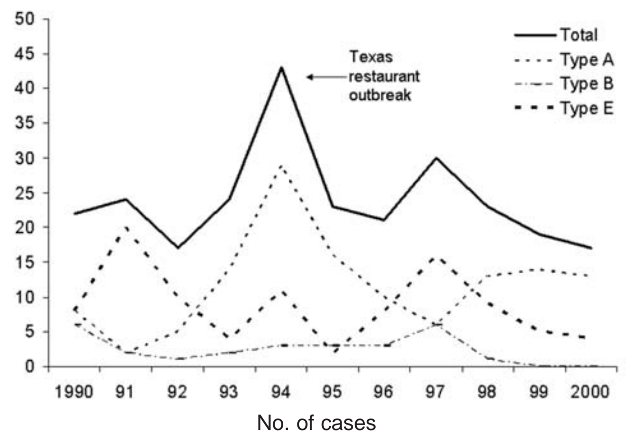

Figure. Foodborne botulism cases in the United States, 1990–2000.

Table 1. States with five or more foodborne botulism cases, 1990–2000

State	No. cases	Incidence/1,000,000[a]
Alaska	103	19
California	37	0.1
Texas	20	0.1
Washington	18	0.3
New Jersey	10	0.1
Georgia	8	0.1
Idaho	7	0.6
Oregon	6	0.2
Ohio	5	<0.1

[a]Using intercensal estimate, U.S. Census Bureau data.

(67%) were caused by homemade foods. Home-canned foods accounted for 47 (69%) of the homemade food events, affecting 70 people, while other types of homemade foods accounted for the remaining 21 (31%) events, which affected 27 people. Of the nine events caused by nonhomemade foods, five (56%) events, which affected 10 people, were caused by commercial foods, and two (22%) events, which affected 25 people, were caused by restaurant-prepared foods.

Improper Food Handling Practices

Improper food handling practices that permit germination and growth of *C. botulinum* with subsequent toxin elaboration were identified in events involving noncanned homemade foods. For example, salsa made with raw vegetables that were placed in nonrefrigerated airtight plastic containers, which likely fostered an anaerobic environment, was associated with events in 1990 and 1993; each event involved one case-patient. Home-bottled garlic-in-oil was associated with events in 1991 in California and in 1999 in Florida. In one of these events, the garlic-in-oil was prepared by using home-canning methods; the mixture was heated to a temperature insufficient to kill *C. botulinum* spores (11). Lastly, investigators have suggested that failure to refrigerate freshly made home-cooked foods not normally associated with botulism, such as the beef chili implicated in an outbreak affecting two persons in New Jersey in 2000, can result in toxin production and illness.

Improper food handling practices identified in the five events caused by commercially produced foods (Table 3) include the following. First, an outbreak of botulism type E, affecting three people, was caused by mohola, a salt-cured traditional Egyptian fish product, reportedly purchased at an ethnic New Jersey retail fish market in 1992 (12). Second, an event of botulism type B, which affected one person in Oregon in 1997, was caused by a commercially produced burrito purchased at a roadside store. Third, an event of botulism type A, which affected one person in California in 1994, was attributable to a commercially produced bean dip that was stored in an airtight plastic container at room temperature; the "keep refrigerated" instruction on the container was in minuscule print. Fourth, an outbreak of botulism type A, which affected two persons in California in 1994, was caused by a commercially produced clam chowder. The chowder was a low-acid product packed in a sealed plastic bag. Though the product was perishable and was sold in the refrigerated section of a grocery store, it carried no "perishable" label with instructions to refrigerate, as required by California state law. The case-patients stored the soup at room temperature for several weeks and then ate it despite its bad odor and flavor. Fifth, an outbreak of botulism type B, affecting three persons in Hawaii in 1990, was caused by commercially caught, retail-sold palani (surgeon fish). The fish was bought fresh, grilled, and eaten immediately; severe illness appeared to be associated with eating the cooked fish intestines (13).

Two outbreaks caused by restaurant-prepared foods involved skordalia, a potato-based dip, and a commercially canned cheese sauce. The first outbreak occurred in Texas in 1993 and affected 17 persons. The skordalia was prepared with potatoes that were baked in aluminum foil wrapping, then left wrapped in the foil at ambient temperature for several days (14). The second outbreak occurred in Georgia in 1993 and affected eight persons. It involved canned cheese sauce eaten after the opened can was left in the restaurant without refrigeration (15).

Botulinum Toxin Types

During the period under study, 60 events were caused by type A botulinum toxin. The implicated foods in 40 (50%) were home-canned products (27 vegetable items, two home-canned soups, two home-canned tuna items, one each home-canned tomato juice, garlic-in-oil, and stew). Other implicated home-prepared foods included five events from potato salad or potatoes, four from homemade soup, two from homemade sausage, and one from each of the following: roast beef, liver paté, bread pudding, salsa, apple pie, hamburger, and chili. In 18 (23%) events caused by botulinum toxin type A, the specific food vehicle was not identified.

During the period under study, 15 events were caused by type B botulinum toxin. The implicated foods in five (33%) of the type B toxin events were home-canned products (corn, eggs, green beans, olives, and an unspecified vegetable), and one event was due to each of the following: a commercially manufactured burrito, commercially caught and sold fish, pasta sauce and meatballs, salsa, turnip greens, and peyote. In four (25%) events, the food vehicle was not identified.

Three events, all attributable to consumption of fish, were caused by botulinum toxin type E. In one event, the toxin type was not identified.

Table 2. Toxin types of foodborne botulism cases and events in the United States, 1990–2000

Toxin type	No. cases (%)	No. events (%)
A	130 (50)	80 (50)
B	27 (10)	23 (14)
E	97 (37)	52 (33)
F	3 (1)	3 (1)
Unknown	6 (2)	5 (2)
Total	263 (100)	160 (100)

Alaska

During the period under study, 58 botulism events occurred in Alaska; 103 persons were affected, with a median of 5 events (range 0–15) and 8 cases (range 0–20) occurring per year. The median number of cases per event was 1 (range 1–5). Most events occurred in the spring through the fall, with a sharp peak in July. The median age of case-patients was 44 years (range 8–92), and 70 (68%) were female. The case-fatality rate was 3%. The contaminated food was identified in 49 (84%) of events. All identified foods were homemade Alaska Native foods. Eleven events, affecting 21 persons, were caused by foods consisting of fermented aquatic mammal tissues, such as muktuk (whale skin and blubber), beaver tail, and seal flipper. Fourteen events, affecting 20 persons, were caused by seal oil; 14 events, affecting 28 persons, were caused by fish foods such as fermented salmon heads and whitefish; 7 events, affecting 18 persons, were caused by fermented fish eggs; and 3 events, affecting 5 persons, were caused by foods with mixed ingredients. Forty-nine (84%) events and 91 (88%) cases were caused by toxin type E, all involving foods from aquatic animals. Eight (14%) events with 11 (11%) cases were caused by toxin type B; 6 of these events were caused by animal foods of aquatic origin, and two were caused by unknown foods. In one event caused by fermented seal, the toxin type was not identified.

U.S. Foodborne Botulism Cases with No Food Vehicle Identified

During the period under study, 37 cases of botulism were reported by state health departments as foodborne botulism attributable to an unknown food; these represented 14% of all cases. Of these, six were associated with outbreaks of two or more cases and therefore can be considered with certainty to be foodborne. The characteristics of the remaining 31 sporadic cases reported as foodborne, but not having an identified food, resembled those of other foodborne botulism cases. Twenty-two (61%) patients were female, and the median age was 55 (range 42–84). Seventeen (55%) cases were caused by toxin type A, 6 (19%) by toxin type B, 5 (16%) by toxin type E, and 3 (10%) by toxin type F. One (3%) case-patient died.

Discussion

In previous eras, conditions conducive to the survival of *C. botulinum* spores and their subsequent germination in food were much more common. Nevertheless, enduring

Table 3. Foodborne botulism events, United States, 1990–2000[a]

Type of processing/food	No. events[b]	No. cases
Noncommercial,home canned		
Asparagus	9	14
Squash	1	2
Peppers	2	4
Corn	1	1
Beans	2	3
Pumpkin	1	1
Greens	2	2
Tomato juice	1	1
Olives	4	4
Beets	3	6
Not specified	3	4
Vegetables, no further specificity	3	3
Mushrooms	2	2
Soup, no further specificity	1	2
Stew, no further specificity	1	3
Potatoes	1	1
Tuna	2	4
Eggplant	1	2
Turnips	1	1
Carrots	1	3
Eggs	1	1
Chiles	1	1
Pickles	1	1
Garlic in oil	2	4
Total	47	70
Noncommercial, not home canned		
Sausage	2	3
Salsa	2	2
Potato salad	2	3
Bread pudding	1	1
Liver paté	1	1
Soup, no further specificity	4	4
Beef chili	1	2
Meatballs and sauce	1	1
Roast beef	1	1
Apple pie	1	1
Potatoes	3	3
Hamburger	1	1
Pickled herring	1	4
Total	21	27
Commercial		
Salted,uneviscerated fish (mohola)	1	3
Palani (surgeon fish)	1	3
Burrito	1	1
Clam chowder	1	2
Bean dip	1	1
Total	5	10
Restaurant-made		
Cheese sauce	1	8
Skordalia potato dip	1	17
Total	2	25

Table 3 continued. Foodborne botulism events, United States, 1990–2000[a]

Type of processing/food	No. events[b]	No. cases
Other		
Peyote tea	1	1
Unknown	25	26
Total, contiguous states and Hawaii	102	160
Alaska		
Noncommercial		
Seal oil	14	20
Fish eggs	7	18
Fermented sea mammals	11	21
Fermented fish	14	28
Mixed ingredients	3	5
Total	49	92
Unknown	913	11
Total, Alaska	58	103
Total, United States	160	263

[a]Foods were implicated either by isolation of toxin from the food or by epidemiologic investigation without laboratory confirmation.
[b]Event was defined as a sporadic case or outbreak of $\geq$2 related cases.

methods of preparing certain homemade foods, new ways of packaging commercial foods, new food preferences, or new techniques for preparing familiar foods that support the growth of *C. botulinum* render it likely that foodborne botulism will afflict humans for the foreseeable future.

From 1990 to 2000, home-canned foods remained a leading cause of foodborne botulism in the United States. New interventions should be explored to ensure that methods of home canning vegetables incorporate adequate barriers to prevent *C. botulinum* germination. Possible areas of research may include development of practical dye indicators for pH and temperatures above those of refrigeration. Botulism associated with Alaska Native foods is likely an age-old problem, compounded in recent decades by altering traditional practices in an unsafe manner, in particular, to include the use of plastic or glass containers for fermentation. To address this problem, the Alaska State Department of Health and Social Services, in partnership with CDC, has developed culturally appropriate educational materials on safer native food preparation. The preventive message focuses on handwashing, using safer, traditional fermentation processes, avoiding plastic and glass containers, shielding fermenting food from sunlight, boiling native foods before consumption, and discarding suspicious foods (www.phppo.cdc.gov/phtn/botulism/default/default.asp) (16).

C. botulinum rarely causes illness because the confluence of conditions required for its germination and toxin production—low acidity, high water activity, absence of preservatives, ambient temperature, and anaerobic milieu—rarely occurs in foods (4). However, from 1990 to 2000, preservative-free, low-salt, commercially produced foods packaged in air-tight containers with no intrinsic barriers to *C. botulinum* spore germination were popular, possibly because they are perceived by the public as healthier. The sole barrier to *C. botulinum* germination in these foods is refrigeration. The outbreaks attributable to commercial bean dip and clam chowder exemplify the failure of this lone safeguard. Prevention could be enhanced by mandating multiple barriers to spore germination, such as acidification and reduced water activity, along with prominent refrigeration instructions, in all low-oxygen packaged commercial foods.

Botulism from traditional recipes of uneviscerated fish is well documented in such preparations as Egyptian fasikh (17) and East-European kapchunka (18). The 1992 outbreak from moloha (Table 3) falls into this category. Current regulations prohibit the sale of uneviscerated fish (19). The outbreak from palani in Hawaii in 1990 is novel because the commercially caught fish was purchased fresh at a retail establishment, cooked, and served immediately (13). Control measures include appropriate refrigeration between catch and sale, similar to those used to control scombroid poisoning (20). From 1990 to 2000, restaurant-associated outbreaks continued to cause a disproportionate number of botulism cases. Investigation of the skordalia dip outbreak identified the practice of leaving baked potatoes wrapped in foil at ambient temperature as the principal hazard; this practice must be completely eliminated from restaurants and homes. Control measures depend on the education of restaurant operators and enforcement by sanitation authorities.

Thirty-one reported sporadic foodborne cases had no identified contaminated food source. Were these, in fact, cases of foodborne botulism? The median age, gender distribution, and fatality rate were similar to those of other foodborne cases, and no patients were reported to be injection drug users, the leading risk factor for wound botulism (21). However, some of these may represent cases of adult intestinal toxemia botulism (AITB) or adult-infant botulism. The diagnosis of AITB is difficult to establish because it requires proof of in vivo toxin production by demonstrating botulinum toxin or *C. botulinum* in patient specimens over time (22). Absence of a toxin-containing food, identification of *C. botulinum* in stool, and a history of abnormal bowel anatomy or recent antimicrobial drug use support the diagnosis of AITB (23,24). Some AITB cases may have been misclassified as foodborne. All three cases of botulism type F reported from 1990 to 2000 were reported as foodborne but with no implicated food. Since identification of type F toxin in food is practicable (25,26), failure to implicate a food may indicate these cases were misclassified AITB. For patients with sporadic cases of adult botulism with no implicated food or injection drug history, repeated stool cultures over time may help establish the diagnosis of AITB. As the clinical syndrome of inhalational botulism probably does not differ from that of

foodborne botulism (27), the possibility of aerosol exposure should be considered in otherwise unexplained cases.

Our data have several limitations. Most clinical data were collected at one point, when antitoxin was sought by the treating clinician from CDC or a state health department. Changes in reporting format during the period reported resulted in incomplete data for a substantial portion of cases. Differences in toxin type–specific rates of intubation and deaths on a subset of cases must be cautiously interpreted in light of these facts.

Foodborne botulism, while rare, remains a public health emergency because of its severity and epidemic potential. Home-canned foods and Alaska Native foods remain the leading causes in the United States, and restaurant-associated outbreaks continue to account for a disproportionate number of illnesses. All suspected cases of botulism should be reported to public health authorities immediately. Prompt epidemiologic investigation helps prevent additional cases and can identify new risk factors for intoxication.

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Computer Algorithms To Detect Bloodstream Infections

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We compared manual and computer-assisted bloodstream infection surveillance for adult inpatients at two hospitals. We identified hospital-acquired, primary, central-venous catheter (CVC)-associated bloodstream infections by using five methods: retrospective, manual record review by investigators; prospective, manual review by infection control professionals; positive blood culture plus manual CVC determination; computer algorithms; and computer algorithms and manual CVC determination. We calculated sensitivity, specificity, predictive values, plus the kappa statistic (κ) between investigator review and other methods, and we correlated infection rates for seven units. The κ value was 0.37 for infection control review, 0.48 for positive blood culture plus manual CVC determination, 0.49 for computer algorithm, and 0.73 for computer algorithm plus manual CVC determination. Unit-specific infection rates, per 1,000 patient days, were 1.0–12.5 by investigator review and 1.4–10.2 by computer algorithm (correlation r = 0.91, p = 0.004). Automated bloodstream infection surveillance with electronic data is an accurate alternative to surveillance with manually collected data.

Central-venous catheter (CVC)-associated bloodstream infections are common adverse events in healthcare facilities, affecting approximately 80,000 intensive-care unit patients in the United States each year (1,2). These infections are a leading cause of death in the United States (3) and are also associated with substantially increased disease and economic cost (4).

As part of an overall prevention and control strategy, the Centers for Disease Control and Prevention's (CDC) Healthcare Infection Control Practices Advisory Committee recommends ongoing surveillance for bloodstream infection (2). However, traditional surveillance methods are dependent on manual collection of clinical data from the medical record, clinical laboratory, and pharmacy by trained infection control professionals. This approach is time-consuming and costly and focuses infection control resources on counting rather than preventing infections. In addition, applying CDC case definitions requires considerable clinical judgment (5), and these definitions may be inconsistently applied. Further, human case finding can lack sensitivity (6), and interinstitutional variability in surveillance techniques complicates interhospital comparisons (7). With the increasing availability of electronic data originating from clinical care (e.g., microbiology results and medication orders), alternative approaches to adverse event detection have been proposed (8) and hold promise for improving detection of bloodstream infections. We present the results of an evaluation study comparing traditional, manual surveillance methods to alternative methods with available clinical electronic data and computer algorithms to identify bloodstream infections.

Methods

The study was conducted at two institutions, both of which participate in the Chicago Antimicrobial Resistance Project: Cook County Hospital, a 600-bed public teaching hospital and Provident Hospital, a 120-bed community hospital. As part of the project, we created a data warehouse by using data from the admission and discharge, pharmacy, microbiology, clinical laboratory, and radiology department databases (9). The data warehouse is a relational database that allows us to link data for individual patients from these separate departments. Data are downloaded from the various departmental databases to our warehouse once every 24 hours; therefore, the algorithms can be applied to clinical data from the previous day.

Facility-specific procedures exist for acquiring and pro-

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cessing blood specimens. At both hospitals, the decision to obtain blood cultures was determined solely by medical providers, without input from infection control professionals or study investigators. After CVC removal, the decision to send a distal segment of the CVC for culture was at the discretion of the medical care provider; both microbiology laboratories accepted these specimens for culture. Since considerable interfacility variability likely exists in CVC culture practices beyond Cook County and Provident Hospitals, we decided not to incorporate these culture results into our computer algorithms.

Blood cultures were obtained and processed at Cook County and Provident Hospitals by using similar methods. At Cook County Hospital, blood cultures were obtained by resident physicians or medical students. At Provident Hospital, blood cultures were obtained by phlebotomists outside of the intensive-care units and by a nurse or physician in the intensive-care unit. At each hospital, blood cultures were injected into Bactec (Becton Dickinson, Inc., Sparks, MD) bottles and incubated for up to 5 days in an automated blood culture detection system. When microbial growth was detected, samples were spread onto solid media and incubated overnight.

Using data from several sources, we compiled a list of all patients who had a positive blood culture hospitalized on inpatient units other than the pediatric or neonatal units from September 1, 2001, through February 28, 2002 (study period). Positive blood cultures obtained <2 days after hospital admission and not evaluated by an infection control professional were excluded. Positive blood cultures obtained within 5 days of the initial positive blood culture were considered as part of the same episode; i.e., these blood cultures were considered polymicrobial infections. At Cook County Hospital, we studied a random sample of positive blood cultures. At Provident Hospital, since a relatively small number of cultures were obtained during the study period, we evaluated all positive blood cultures. Approval was obtained by the local and CDC human participant review boards.

Investigator Review

We used the CDC definition for primary, CVC-associated, laboratory-confirmed bloodstream infection (10). Four study investigators, all of whom had previous experience applying these definitions, performed retrospective medical record reviews. Two investigators independently reviewed each medical record. If there was a judgment disagreement between the two investigators, a third reviewer categorized the blood culture. Investigators were blinded to other investigators' reviews and to determinations made by review and by computer algorithms. To minimize the likelihood of investigator interpretation approximating the computer algorithm, i.e., systematic bias in definition interpretation, the details of the computer algorithms were not disclosed to three of the four reviewers. The reviewer who participated in the construction of the computer algorithms functioned in the same capacity as the other three reviewers (i.e., all four reviewers could participate in the initial or final reviews).

Infection Control Professional Review

During the study period, infection control professionals at Cook County and Provident Hospitals performed prospective hospitalwide bloodstream infection surveillance using the CDC definitions (10). Six infection control professionals submitted data, four at Cook County Hospital and two at Provident Hospital; all were registered nurses and had 10–30 years of infection control experience. All six had attended a 1-day surveillance seminar conducted by CDC personnel and had access to an infection control professional who had attended a CDC-sponsored infection surveillance training course; four were certified in infection control.

At Cook County Hospital, a list of all positive blood culture results was generated by a single person in the microbiology laboratory. Duplicates (i.e., the same species identified within the previous 30 days) were excluded, and the list was distributed to the infection control professionals. For those patients who had not been discharged, the infection control professionals reviewed the medical chart, and if their assessment differed from the medical record documentation, they could discuss the case with the medical team. For patients who had been discharged, only the medical record was reviewed. For polymicrobial cultures (i.e., >1 organism isolated from a blood culture), infection control professionals categorized each isolate. The infection control professionals did not participate in the medical team's ward rounds. At Provident Hospital, the procedures were similar except that the laboratory printed out all positive culture results, and the infection control professional manually excluded duplicate results.

Determinations were recorded on a standardized, scannable form, and the forms were sent to a central location, where they were evaluated for completeness and then scanned into a database. In cases where the infection control professional did not record whether the infection was hospital- or community-acquired, we categorized the infection as hospital-acquired if it was detected >2 days after hospital admission.

Computer Algorithms

We evaluated several methods to categorize blood cultures. First, we evaluated a simple method that required only a computer report of a positive blood culture recovered >2 days after admission plus manual determination of whether a CVC was present.

Table 1. Computer algorithms and corresponding NNIS system definitions to categorize blood culture isolates, September 2001–February 2002, Cook County Hospital, Chicago, Illinois[a]

Determination[b]	Computer rule	NNIS definitions
Hospital acquired	(A) Acquired blood culture ≥3 days after hospital admission	No evidence infection present or incubating at time of hospital admission, unless infection was related to previous admission to this hospital
Infection	(B1) Microbiology data: pathogen other than CSC[c] cultured from blood, or ≥2 CSC isolates recovered from blood within 5 days of initial positive blood culture (B2) Microbiology and pharmacy data: pathogen cultured from blood or ≥2 CSC[d] isolates within 5 days of initial positive blood culture, or CSC cultured from blood once and vancomycin administered within 3 days before until 1 day after isolate identification	Patient has at least one sign or symptom: fever (>38°C), chills, or hypotension and at least one of the following: pathogen cultured from ≥1 blood cultures, CSC cultured from ≥2 blood cultures drawn on separate occasions, CSC cultured from at least 1 blood culture from patient with intravenous line, and physician institutes appropriate antimicrobial drug therapy
Secondary bloodstream infection (BSI)[d]	(C1) Time restricted: organism recovered from blood also recovered from a nonblood culture, 3–7 days after the blood culture acquisition date[d] (C2) Length of stay: organism recovered from blood also recovered from a nonblood culture during the entire length of stay[d]	The organism cultured from the blood is related to an infection at another site
Central-venous catheter (CVC) associated[e]	(D) No algorithm developed, all BSI were considered CVC associated	Vascular access device that terminated at or close to heart or one of great vessels within the 48-hour period before BSI developed

[a]NNIS, National Nosocomial Infection Surveillance; CSC, common skin contaminant; BSI, bloodstream infection.
[b]For each determination, if the computer rule or NNIS definition was not met, the isolate was considered as one the following: community acquired, a contaminant, primary BSI, or not CVC associated.
[c]We used the examples of CSCs listed in the NNIS manual: diphtheroids, *Bacillus* spp., *Propionibacterium* spp., coagulase-negative staphylococci, or micrococci.
[d]Catheter tip and stool cultures were excluded for both algorithms. CSCs had to be cultured from a wound for the BSI to be considered as a secondary BSI.
[e]Includes tunneled or nontunneled catheters inserted into the subclavian, jugular, or femoral veins; pulmonary artery catheters; hemodialysis catheter; totally implanted devices (ports); peripherally inserted central catheters; and introducer sheaths.

Second, after consultation with infectious disease clinicians, we developed rules that were combined into more sophisticated computer algorithms (Table 1, Figure 1). Two rules were developed for two of the determinations that were required. For determining infection versus contamination, rule B1 used only microbiology data, while rule B2 used microbiologic and pharmacy data. For determining primary versus secondary (i.e., the organism cultured from the blood is related to an infection at another site) bloodstream infection, rule C1 was limited to a 10-day window, while rule C2 extended throughout the hospitalization. Since two options existed for two separate rules, these rules were combined into four separate algorithms. We report the results of the algorithms that had the best (rules A, B2, C2, and D) and worst (rules A, B1, C1, and D) performance. Consistent with the manual methods, polymicrobial cultures were considered a single event. Polymicrobial blood cultures were considered an infection if any isolate recovered from the same culture met infection criteria, and, in contrast to the manual methods, were considered a secondary bloodstream infection if any isolate that met infection criteria also met criteria to be classified as secondary. Third, since we could not automate CVC detection, we also evaluated augmentation of automated bloodstream infection detection with manual determination of a CVC.

For Provident Hospital, since the number of positive blood cultures evaluated by each rule was relatively small, we do not report the performance characteristics. We do report the results for the best and worst computer algorithms at each hospital and at both hospitals combined.

Statistics

For polymicrobial cultures, we analyzed the results at the level of the blood culture. We were primarily interested in evaluating the detection of hospital-acquired, primary, CVC-associated bloodstream infections; therefore, by investigator or infection control professional review, if any isolate from a polymicrobial culture met the necessary criteria, the blood culture was classified as a hospital-acquired, primary, CVC-associated bloodstream infection.

We present the results of comparisons for the blood cultures that were evaluated by all methods. For calculation of sensitivity, specificity, and predictive values, we considered the investigator review to be the reference standard. Next, we calculated the agreement between investigator review and the other methods using the kappa statistic (κ) (11). Since all organisms that were not common skin commensals were considered an infection, we included only common skin commensals to evaluate the rule distinguishing infection versus contaminant.

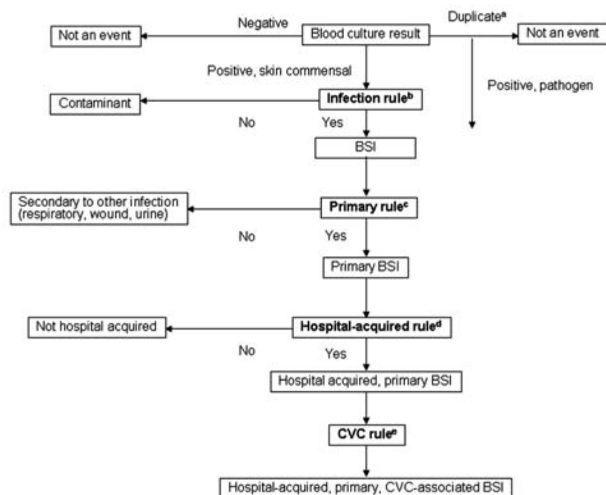

Figure 1. Flowchart displaying the determinations necessary for categorizing positive blood culture by computer algorithm. The rules described in Table 1 are in bold. Blood cultures were obtained from patients at Cook County and Provident Hospitals, September 1, 2001–February 28, 2002, Chicago, Illinois. BSI, blood stream infection; CVC, central-venous catheter. [a]Same species isolated from blood within 30 days. [b]Rule B1 or B2 (Table 1). [c]Rule C1 or C2 (Table 1). [d]Rule A (Table 1). [e]Rule D (Table 1).

We report bloodstream infection rates per 1,000 patient-days for certain units in the hospital. Hospital units were aggregated according to the type of patient-care delivered, as identified by hospital personnel. For example, data from all nonintensive care medical wards were aggregated. Also, because of the relatively low number of patient-days in the burn, trauma, and neurosurgical intensive-care units (ICU), we aggregated the bloodstream infection rates for these units and report them as specialty ICUs. We calculated the Pearson correlation coefficient for bloodstream infection rates determined by investigator review versus other methods, stratified by hospital unit. Since only a sample of blood cultures was evaluated at Cook County Hospital, the rates were adjusted to account for the unit-specific sampling fraction. We also calculated the Pearson correlation coefficient, comparing the number of bloodstream infections per month identified by investigator review versus the other methods. All analyses were performed by using SAS statistical package version 8.02 (SAS Institute Inc., Cary, NC).

Results

At Cook County Hospital, 104 positive blood cultures from 99 patients were evaluated by all methods (Figure 2A). Of the 99 patients, most were male (58%) and were cared for in non-ICUs (65%); the median patient age was 52 years. Of the 104 patients with positive blood cultures, 83 (79%) were determined to have infection by investigator review, 55 (53%) had primary bloodstream infection, and 39 (37.5%) had hospital-acquired, primary, CVC-associated bloodstream infection. The most common organisms were coagulase-negative staphylococci (n = 45), *Staphylococcus aureus* (n = 23), *Enterococcus* spp. (n = 11), *Pseudomonas aeruginosa* (n = 4), *Escherichia coli* (n = 4), and *Candida albicans* (n = 4); nine (8.7%) infections were polymicrobial.

At Provident Hospital, 40 positive blood cultures were eligible for investigator review; 31 cultures from 28 patients were evaluated by all methods (Figure 2B). Of the 28 patients, most were male (54%) and cared for in non-ICUs (68%); the median patient age was 60 years. Of the 31 patients whose cultures were evaluated by all methods, 29 (94%) were determined to have infection by investigator review, 17 (55%) were primary, and 9 (29%) were hospital-acquired, primary CVC-associated bloodstream infection. The most common organisms were *S. aureus* (n = 9), coagulase-negative staphylococci (n = 6), *Enterococcus* spp. (n = 5), *P. aeruginosa* (n = 3), *E. coli* (n = 2), and *C. albicans* (n = 2); no polymicrobial infections occurred.

Hospital versus Community-acquired Rule

When we evaluated the hospital versus community-acquired rule at Cook County Hospital, the computer rule A had a slightly higher sensitivity, specificity, and κ statistic than did the infection control professional review (Table 2). Only one computer rule was evaluated (Table 1).

Infection versus Contamination Rule for Common Skin Commensals

At Cook County Hospital, infection control professional review and computer rule B2 (which used microbiologic and pharmacy data) had similar performance (Table 2). Computer rule B1 (which used only microbiologic data) was less sensitive (55%) but had a similar κ (0.45).

Primary versus Secondary Rule

At Cook County Hospital, infection control professional review and computer rule C2 had similar sensitivities, specificities, and κ statistics. Both determinations had limited specificity, i.e., some secondary infections were misclassified as primary bloodstream infections. The 12 infection syndromes classified as primary by computer algorithm and secondary by investigator review were lower respiratory tract (n = 5, 42%), intraabdominal (n = 3, 25%), skin or soft tissue (n = 2, 17%), or surgical site (n = 2, 17%); no urinary tract infection was misclassified as a primary bloodstream infection by computer algorithm. Computer rule C1, which evaluated only culture results within a time frame around the blood culture acquisition date, had lower specificity than rule C2 (data not shown).

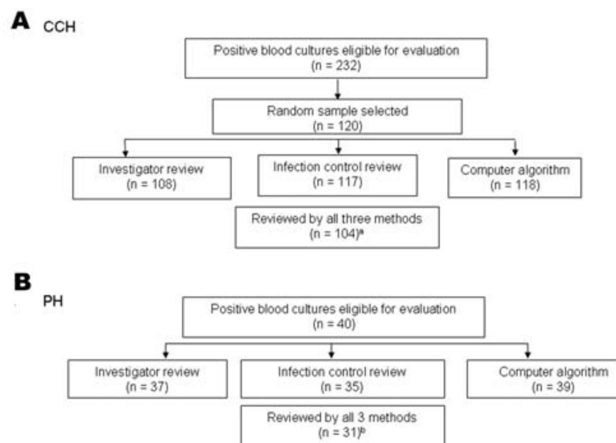

Figure 2. Flowchart displaying the number of blood cultures eligible for evaluation and the number evaluated by investigator review, infection control professional review, and computer algorithm at A) Cook County Hospital (CCH) and B) Provident Hospital (PH), September 1, 2001–February 28, 2002, Chicago, Illinois. [a]At CCH, 12 medical records were unavailable for investigator review; three positive blood cultures were not evaluated by an infection control professional; and two positive blood cultures did not have culture dates stored electronically and, thus, were inaccessible to the computer algorithm. [b]At PH, three medical records were unavailable for investigator review, five positive blood cultures were not evaluated by an infection control professional, and one positive blood culture was not in the data warehouse (this blood culture isolate also was not documented in the medical record).

Bloodstream Infection Algorithm

For overall ability to detect hospital-acquired, primary, CVC-associated bloodstream infection, we found that the simplest method (computer determination of a positive culture plus manual CVC determination) performed better than infection control professional review (κ = 0.48 vs. κ = 0.37, Table 3). The best and worst performing computer algorithms had good performance (κ = 0.49 and κ = 0.42, respectively). When manual determination of a CVC was added to the best performing computer algorithm, the correlation was significantly better than the infection control professional review (κ = 0.73, p = 0.002). At each hospital, the best performing computer algorithm, with or without manual CVC determination, performed better than infection control professional review. For both hospitals combined, the number of hospital-acquired, primary, CVC-associated bloodstream infections varied by method, investigator review (n = 48), infection control professional review (n = 56), positive culture plus manual CVC determination (n = 86), computer algorithm (n = 64), and computer algorithm plus manual CVC determination (n = 48).

Comparison of the Monthly Variation

At Cook County Hospital, when the number of hospital-acquired, CVC-associated bloodstream infections per month was considered, infection control professional review (r = 0.71) was not as well correlated with investigator review as the computer algorithm (r = 0.89) was (Figure 3). When we augmented the computer algorithm with manual CVC determination, the effect was minimal on the correlation between the monthly variations (data not shown). At Provident Hospital, the monthly number of bloodstream infections was too small to provide meaningful comparisons.

Comparisons of Unit-Specific Bloodstream Infection Rates

At Cook County Hospital, the patient care unit–specific bloodstream infection rates determined by investigator review versus those determined by computer algorithm had the same rank from highest to lowest: surgical intensive care, medical intensive care, HIV ward, surgical wards, specialty intensive care, step-down units, and medical wards (Figure 4). The bloodstream infection rates were well correlated between the investigator review and the computer algorithm or infection control professional review. At Provident Hospital, the bloodstream infection rates per 1,000 patient days were as follows: on the non-

Table 2. Positive blood cultures as categorized by computer rules or infection control professional (ICP) review, compared to investigator review,[a] Cook County Hospital, Chicago, IL

Determination	Method	No. cultures[c]	Sensitivity (%)	Specificity (%)	κ
Hospital vs. community acquisition	Computer rule A	77	97	73	0.74
	ICP review	77	94	67	0.62
Infection vs. contamination[d]	Computer rule B2[e]	43	77	71	0.49
	ICP review	43	77	76	0.53
Primary vs. secondary	Computer rule C2[f]	76	90	57	0.49
	ICP review	76	83	64	0.48

[a]Our reference standard.
[b]The single best rule for each determination is displayed.
[c]Since all determinations were not made for each blood culture, e.g., contaminants often were not further categorized, the number of cultures evaluated varies.
[d]Infection determination is presented for common skin commensals only; other organisms were considered as infections; see Table 1 for definitions.
[e]Rule B2 evaluated microbiology and pharmacy results; see Table 1.
[f]Rule C2 evaluated microbiology results during the entire length of stay; see Table 1.

Table 3. Comparing alternative methods for determining if positive blood cultures represented a hospital-acquired, primary, central-venous catheter–associated bloodstream infection, Cook County and Provident Hospitals, Chicago, Illinois[a]

Method	% sensitivity	% specificity	% PVP	% PVN	κ (95% CI)
Cook County Hospital (n = 104)					
Investigator review (reference method)	–	–	–	–	–
Infection control professional review	67	75	62	79	0.41 (0.24–0.59)
Positive blood culture + CVC determination[b]	100	55	57	100	0.48 (0.35–0.62)
Worst computer algorithm (rules A, B1, C1, D)[c]	72	74	62	81	0.44 (0.27–0.62)
Best computer algorithm (rules A, B2, C2, D)[d]	79	72	63	85	0.49 (0.33–0.66)
Computer algorithm + CVC determination[b]	79	88	79	88	0.67 (0.52–0.82)[e]
Provident Hospital (n = 31)					
Investigator review (reference method)	–	–	–	–	–
Infection control professional review	56	68	42	79	0.22 (–0.13–0.56)
Positive blood culture + CVC determination[b]	100	59	50	100	0.46 (0.20–0.70)
Worst computer algorithm (rules A, B1, C1, D)[c]	78	64	53	88	0.35 (0.04–0.65)
Best computer algorithm (rules A, B2, C2, D)[d]	89	68	53	94	0.48 (0.19–0.76)
Computer algorithm + CVC determination[b]	89	95	89	95	0.84 (0.63–1.0)[e]
Summary for both hospitals (n = 135)					
Investigator review (reference method)	–	–	–	–	–
Infection control professional review	65	74	57	79	0.37 (0.21–0.53)[e]
Positive blood culture + CVC determination[b]	100	56	56	100	0.48 (0.36–0.60)
Worst computer algorithm (rules A, B1, C1, D)[c]	72	74	62	81	0.42 (0.27–0.57)
Best computer algorithm (rules A, B2, C2, D)[d]	81	72	62	87	0.49 (0.35–0.63)
Computer algorithm + CVC determination[b]	81	90	81	90	0.73 (0.61–0.85)[e]

[a]PVP, predictive value positive; PVN, predictive value negative, CI, confidence interval.
[b]Presence of a central-venous catheter (CVC) determined by investigator medical record review.
[c]The computer algorithm with the worst agreement, which used only microbiology data for the determination of infection vs. contaminant (rule B1, Table 1), and an abbreviated time period for the determination of primary vs. secondary (rule C1, Table 1).
[d]The computer algorithm with the best agreement, which used the microbiology and pharmacy data for the determination of infection vs. contaminant (rule B2, Table 1), and the entire length of stay for the determination of primary vs. secondary (rule C2, Table 1).
[e]Agreement between investigator review and the best performing computer algorithm plus CVC determination was significantly better than between investigator and infection control professional reviews, i.e., p value < 0.05.

ICUs, investigator review = 0.41, infection control professional review = 0.39, and computer algorithm = 0.62; in the ICUs, investigator review = 2.05, infection control professional review = 3.68, and computer algorithm = 3.68.

Discussion

We used electronic data from clinical information systems to evaluate the accuracy of computer algorithms to detect hospital-acquired primary CVC-associated bloodstream infections. Compared with investigator chart review (our reference standard), we found that computer algorithms that used electronic clinical data outperformed manual review by infection control professionals. When the computer algorithm was augmented by manually determining whether a CVC was present, agreement with investigator review was excellent. These results suggest that automated surveillance for CVC-associated bloodstream infections by using electronic data from clinical information systems could supplement or even supplant manual surveillance, which would allow infection control professionals to focus on other surveillance activities or prevention interventions.

CDC's National Nosocomial Infection Surveillance (NNIS) system reports CVC-associated, hospital-acquired, primary bloodstream infection rates. Determining whether a bloodstream infections is primary and catheter-associated is worthwhile because some prevention strategies differ for catheter-associated versus secondary bacteremias; e.g., the former can be prevented through proper catheter insertion, maintenance, and dressing care (2,12). However, hospital-wide bloodstream infection surveillance at the three Cook

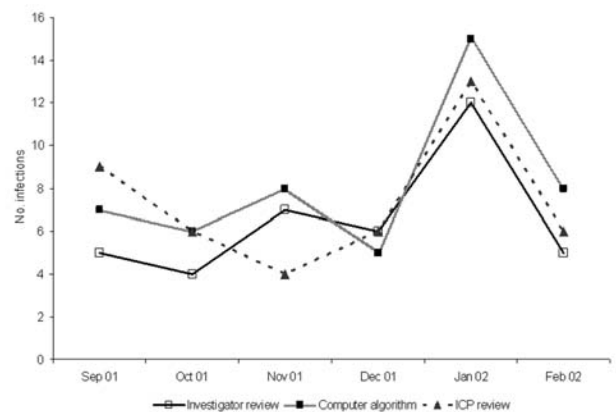

Figure 3. Display of the monthly number of hospital-acquired, primary, central-venous catheter–associated bloodstream infections (BSIs) determined by separate methods, and correlation of the computer algorithm and infection control professional (ICP) review to the investigator review, Cook County Hospital, Chicago, Illinois. Computer algorithm r = 0.89, p = 0.02; ICP review r = 0.71, p = 0.11.

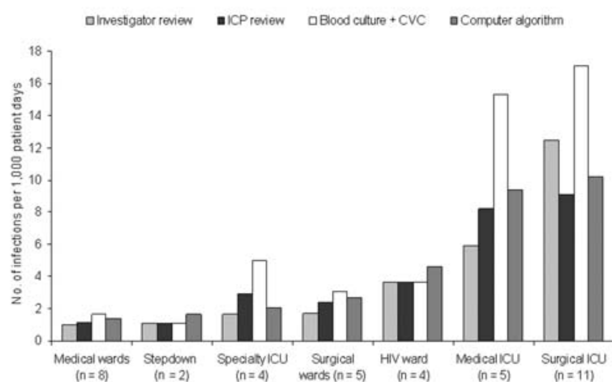

Figure 4. Comparison of the hospital-acquired, primary, central-venous catheter (CVC)-associated bloodstream infection (BSI) rate for adult patient–care units determined by two separate manual methods (i.e., infection control professional [ICP] and investigator review), by positive blood culture plus manual CVC determination, and by computer algorithm, Cook County Hospital, September 1, 2001–February 28, 2002, Chicago, Illinois. The number of hospital-acquired, primary, CVC-associated bloodstream infections determined by investigator review is displayed in parentheses. Correlation coefficient (r) and p value for comparisons between investigator review and each method were as follows: infection control professional review r = 0.95, p = 0.001; blood culture + central line determination r = 0.90, p = 0.006; computer algorithm r = 0.91, p = 0.004. ICU, intensive-care unit.

County Bureau of Health Services hospitals is labor-intensive and estimated to consume, at a minimum, 452 person hours per year (9). This estimate is low because it does not include the time required to identify and list bacteremic patients or record these patients into an electronic database.

Automated infection detection has several advantages, including the following: applying definitions consistently across healthcare facilities and over time, thus avoiding variations among infection control professionals' methods for case-finding and interpretations of the definitions; freeing infection control professionals' time to perform prevention activities; and expanding surveillance to non-ICUs, where CVCs are now common (13).

Since positive blood culture results are central to the bloodstream infection definition and readily available electronically, adapting the bloodstream infection definition is relatively easy for computer algorithms. For other infection syndromes (e.g., hospital-acquired pneumonia), the rules may be more difficult to construct. Despite the relative simplicity of bloodstream infection algorithms, many determinations, or "rules," had to be considered, and various options were considered for each.

The rule for determining hospital versus community acquisition, i.e., a positive blood culture ≥3 days after admission, performed well at Cook County Hospital but poorly at Provident Hospital (data not shown), where some community-acquired bloodstream infections were not detected until ≥3 days after hospital admission. Since some of these positive blood cultures were caused by secondary bloodstream infections, these delays did not adversely affect the performance of the final algorithm, which incorporated additional rules.

The computer rule for determining primary versus secondary bloodstream infection was problematic when the presumed source of these bloodstream infections was not culture-positive, usually for lower respiratory tract infections. We minimized this problem by evaluating nonblood culture results during a patient's length of stay; however, this solution would not be desirable for patient populations with prolonged lengths of stay. The specificity of automated primary bloodstream infection detection could be improved by interpreting radiology reports or using International Classification of Disease codes to automate pneumonia detection (14).

To determine infection versus contamination for common skin commensals by including appropriate antimicrobial use for single positive blood cultures as a criterion for bloodstream infection, we may be evaluating physician prescribing behavior rather than identifying true bloodstream infections; i.e., some episodes of common skin commensals isolated only once are contaminants unnecessarily treated with antimicrobial drugs (15). Since the CDC's bloodstream infection definition includes this criterion, including antimicrobial use in our computer infection rule improved the performance of this algorithm. Despite the potential inaccuracy, reporting the frequency of antimicrobial drug therapy for common skin commensals isolated only once may help healthcare facilities identify episodes of unnecessary drug therapy.

Other investigators have tried to either fully automate infection detection or automate identification of patients who have a high probability of being infected (16–19). These studies demonstrate the feasibility of automated infection detection. Our study adds additional information by comparing a fully automated computer algorithm, a partially automated computer algorithm (including manual CVC determination), and infection control professional blood culture categorization to the investigators' manual evaluation.

Our study has several limitations. Investigator review may have been influenced by knowledge of the computer algorithms; however, three of the four reviewers were not familiar with the details of the computer algorithms. In addition, our evaluation included only patients at a public community hospital or public teaching hospital, and our findings may not be generalizable to other healthcare facilities. In particular, several factors could influence the performance characteristics of both computer algorithm and manual surveillance, including the frequency of blood culture acquisition, CVC use, the distribution of pathogens,

and the proportion of bloodstream infections categorized as secondary. Also, we expected better agreement between investigator and infection control professional reviews. Potentially, agreement could be improved by additional infection control professional training. The computer algorithm could also likely be improved by incrementally refining the algorithm or including additional clinical information. The cost of refining the algorithm with local data or including more clinical data would be a decrease in the generalizability or feasibility of the algorithms. Further, many hospital information systems have not been structured so that adverse event detection can be automated. The algorithms we used could be improved when hospital information systems evolve to routinely capture additional clinical data (e.g., patient vital signs) or process and interpret textual reports (e.g., radiograph reports) (14,20,21).

Reporting data to public health agencies electronically has recently become more common (22,23). One important and achievable patient safety initiative is reducing CVC-associated bloodstream infections (24). Traditional surveillance methods are too labor intensive to allow hospitalwide surveillance; therefore, NNIS has recommended focusing surveillance on ICUs. However, intravascular device use has changed and, currently, most CVCs may be outside ICUs (13). Using electronic data holds promise for identifying some infection syndromes, and hospitalwide surveillance may be feasible. Hospital information system vendors can play a key role in facilitating automated healthcare-associated adverse event detection. Our study demonstrates that to detect hospital-acquired primary CVC-associated bloodstream infections, using computer algorithms to interpret blood culture results was as reliable as a separate manual review. These findings justify efforts to modify surveillance systems to fully or partially automate bloodstream infection detection.

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Space-Time Cluster Analysis of Invasive Meningococcal Disease

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Clusters are recognized when meningococcal cases of the same phenotypic strain (markers: serogroup, serotype, and subtype) occur in spatial and temporal proximity. The incidence of such clusters was compared to the incidence that would be expected by chance by using space-time nearest-neighbor analysis of 4,887 confirmed invasive meningococcal cases identified in the 9-year surveillance period 1993–2001 in the Netherlands. Clustering beyond chance only occurred among the closest neighboring cases (comparable to secondary cases) and was small (3.1%, 95% confidence interval 2.1%–4.1%).

An outbreak of invasive meningococcal disease is a public health emergency because of the disease's unpredictability, sudden lethality, and serious sequelae. Although risk factors are known, the reasons for developing invasive disease are not fully understood. Most persons, when colonized with *Neisseria meningitidis*, become asymptomatic carriers and are sources for further transmission. The apparently sporadic occurrence of invasive disease reflects invisible transmission chains of circulating strains, since invasive disease develops in only a small proportion of those infected. The precise mechanisms generating clusters or outbreaks puzzle public health workers, epidemiologists, and microbiologists (1,2).

During the 9-year period 1993–2001, the Netherlands had a population between 15.3 and 16 million and encompassed 33,900 km^2. Most of the ≈500 annual reports of meningococcal disease were sporadic cases, and serogroup B is the most common. From 1993 to 2001, the number of reported cases was from 422 to 770 per year; the peak occurred in 2001 as a result of an increase in serogroup C meningococcal cases. The mean incidence, based on reports of ≈3.4 per 100,000 per year, is comparable to that in England and Wales (3.7) (3) but three times higher than in the United States (1.1) (4). The Dutch policy for preventing secondary cases compares to the policy in most Western countries and is based on identifying and prophylactically treating close contacts. When two or more possibly related cases (secondary case or cluster) are identified, group contacts in an educational institution (daycare center or primary school) also receive prophylaxis with rifampicin. In the Netherlands, routine vaccination of children for serogroup C meningococcal disease was implemented in September 2002. Furthermore, from June to October 2002, a vaccination campaign was carried out for all 1- to 18-year-olds in response to the increase of serogroup C cases in 2001 and 2002.

Outbreaks are recognized when place (e.g., an educational institution like a primary school), time (e.g., within 1 month), and conventional phenotypic markers (same serogroup, serotype, and subtype) make a connection likely (field cluster) or when an excess of incidence (e.g., 20x normal) is noticed in a retrospectively specified geographic or population area within a chosen period (community outbreak). Field clusters and community outbreaks are rarely seen in the Netherlands, possibly because of underreporting. A group of unrelated cases that occur in temporal and spatial proximity may be misinterpreted as a cluster or outbreak, but these cases would not justify additional public health measures, except perhaps to reassure the public. In a real cluster, cases of the same strain occur in temporal and spatial proximity at a higher frequency than by chance. The objective of our study was to explore the phenomenon of meningococcal clustering in a more objective way by using a nearest-neighbor analysis in space and time that compares the actual occurrence of clusters with their background incidence.

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[1]Deceased January 24, 2004.

Patients and Methods

Data Collection

We used data collected from two surveillance sources: mandatory reports from January 1993 through May 2001 and reports of laboratory-confirmed *N. meningitidis* isolates collected by the Netherlands Reference Laboratory for Bacterial Meningitis in the same period. Additionally, reports of field clusters occurring during the same time were collected as reference.

Reported Cases

Report data were obtained from the Inspectorate of Health Care. According to the Communicable Disease Act, physicians must report cases of meningococcal disease to their Municipal Public Health Service. The case definition for report includes clinical meningococcal disease in combination with microbiologic confirmation: *N. meningitidis* isolated from blood or cerebrospinal fluid (CSF); meningococcal antigen or DNA detected in cerebrospinal fluid by latex agglutination or polymerase chain reaction; or gram-negative diplococci detected in cerebrospinal fluid, blood, or skin biopsy. The following information was available on an individual level: date of birth, gender, initials, postal code, municipality, date of report, date of first symptoms, date of diagnosis, and age at notification.

Laboratory Isolates

The reference laboratory collects meningococcal strains from patients with meningitis or septicemia, isolated from blood or CSF. Strains are sent on a voluntary basis to the reference laboratory by all clinical microbiologic laboratories throughout the country. A strain is defined as an isolate of *N. meningitidis* from a patient. When two strains have the same phenotypic markers (serogroup, serotype, and subtype), these are considered to be identical and to belong to one serosubtype. The following information was available for individual patients: date of birth, gender, initials, municipality, date of sample collection, submitting laboratory, date of receipt of strain, date of blood culture, date of lumbar puncture, source of isolate (blood or CSF), serogroup, serotype, and subtype.

Record Linkage

Records between these two sources were linked (case ascertainment) by using SAS version 8.1 (SAS Institute Inc., Cary, NC). First, records were linked by date of birth, gender, and initials. Records remaining unlinked were then linked by combinations of two variables. The links in the first step were considered correct, while all further links were checked manually for consistency in data fields, spelling mistakes in initials, date of birth, and municipality. In Table 1, we provide an overview of the number of cases and serogroup profile of the data used in our analysis.

Field Cluster

After notification of meningococcal disease, the Municipal Public Health Service considers taking public

Table 1. Overview of meningococcal disease cases and serogroup profile of cases included for analysis, 1993–2001

Characteristic	1993	1994	1995	1996	1997	1998	1999[a]	2000	2001[b]	Total
Reported cases (per 100,000 population)	563 (3.7)	422 (2.7)	460 (3.0)	482 (3.1)	491 (3.2)	505 (3.2)	531 (3.4)	516 (3.3)	770 (4.8)	4,740 (3.4)
Case-ascertainment (per 100,000 population)	753 (4.9)	571 (3.7)	689 (4.5)	659 (4.3)	658 (4.2)	704 (4.5)	597 (3.7)	532 (3.4)	396 (5.9)	5,559 (4.3)
Nonconfirmed cases[c]	115	91	88	94	103	86	44	1	41	663
Meningococcal cases included for analysis	638	480	601	565	555	619	553	531	354	4,896
Serogroup profile[d]										
A	3	2	0	0	2	0	0	0	0	7
B (%)	524 (82)	399 (83)	527 (88)	498 (88)	458 (83)	536 (87)	455 (82)	413 (78)	229 (65)	4,039 (82)
C (%)	101 (16)	65 (12)	57 (11)	57 (11)	81 (15)	72 13)	79 (15)	103 (19)	114 (21)	729 (15)
W135 (%)	4 (1)	5 (1)	7 (1)	3 (1)	6 (1)	4 (1)	12 (2)	12 (2)	7 (2)	60 (1)
X	1	0	0	1	2	1	1	1	0	7
Y	4	5	7	5	6	2	5	2	3	39
Z	0	0	1	0	0	0	0	0	2	3
29E	0	2	0	1	0	1	0	0	0	4
Not serogroupable	1	2	2	0	0	2	1	0	0	8

[a]Case-ascertainment (number of cases after linking procedure) was hampered due to lack of identifying variables from April 1, 1999, when the new Dutch Communicable Disease Act was introduced.
[b]Laboratory data included from January 1993 to May 2001; during the year 2001, the surveillance was more active because of the increase in serogroup C cases.
[c]After linking the reported cases with the laboratory cases, no strain was available for these cases.
[d]Of 4,896 confirmed cases, 9 could not be used in the analysis because of recording errors: 2 serogroup A cases, 4 serogroup B cases, 1 serogroup C, 1 serogroup W135, and 1 serogroup Y.

health measures. Depending on the attentiveness of the communicable disease consultant, field clusters are recognized and reported to the Inspectorate of Health Care, which made this information available for our investigation. Accurate data on actual rifampicin prophylaxis were not available. Field clusters were named after their probable transmission route: family, daycare center, primary school, or swimming pool.

Statistical Analysis

Clustering of meningococcal cases is defined as excess occurrence of the same serosubtype in patients, in spatial and temporal proximity. We used patients' residences as "place" and chose the first day of illness as "time." The actual incidence of clustering was compared to the incidence that would be expected by chance, by using space-time nearest-neighbor analysis (Figure 1). To quantify the phenomenon of clustering, we defined the concept of space-time nearest-neighborship as follows. We defined *nt* nearest-neighbors in time of case 1 as the *n* cases that occur closest (in time) to case 1. Similarly the *np* nearest-neighbors in place of case 1 are the *n* cases that occur closest in space to case 1. The distance between cases is defined as the distance in a straight line between the geographic centers of the reported cases (municipality or postal code area). The *k* cases that are both *nt* nearest-neighbors in time and *np* nearest-neighbors in place (intersection of place and time), are now the group of the 1st, 2nd, …, and *k*th nearest-neighbors (i.e., nearest in both place and time). The order (first, second, and so on) is set in such a way that $k = 1$ defines the first nearest-neighbor, $k = 2$ defines the second nearest-neighbor, and so on. A program was written in C to analyze *k*th nearest-neighborship. This program is available from the authors.

First, we calculated the "background" probability that a *k*th nearest-neighbor is of the same strain, under no clustering as the null hypothesis, by calculating the frequency of having a *k*th nearest-neighbor of the same strain when the observed strains are randomly assigned to the observed dates and places of actual cases. This shuffling is called random labeling (5,6). The null hypothesis assumes complete homogeneity in space and time, which is plausible for small areas within a short time (e.g., 1 year); however, spatial and temporal heterogeneity may give rise to spurious clustering. The prevalence of serogroup B was not constant during the 9 years of our study (Table 1), and the ratio of serogroup B to other serogroups varies somewhat by region (Figure 2). Therefore, this concept of "random labeling" may not apply to our meningococcal data, since it ignores regional differences in occurrence and slow trends in the presence of certain serosubtypes over the period of observation. Thus, random labeling would underestimate the true null (under no clustering) background probability that a nearest-neighbor is of the same strain, thereby overestimating clustering. We decided that the true null background probability is best estimated by the observed frequency of the mean of the 6th to 10th nearest-neighbors (the null probability of no clustering), which assumes clustering is a priori implausible beyond the 5th nearest-neighbor. We calculated 95% confidence intervals (CI) for the excess chance that the first, second, third, fourth, and fifth nearest-neighbor is of the same strain by using paired t-tests. These paired t-tests were carried out on a) the indicator (0/1) variable, indicating whether the first, second, third, fourth, or fifth nearest-neighbor is of the same strain, and b) the average of five such indicator variables for the 6th to 10th nearest-neighbor. The above analyses were calculated for all cases combined but also separately for serogroups B, C, and W135 and for each serosubtype separately.

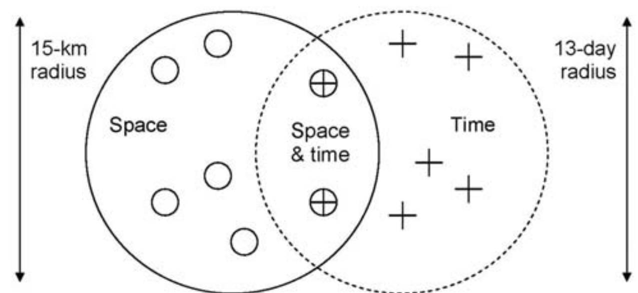

Figure 1. The concept of space-time nearest-neighborship. Nearest-neighbors in space-time are defined as cases that are nearest-neighbors in both space and time. To define the *k*th nearest-neighbors in space-time, we chose the number *n* (e.g., $n = 7$; thus 7 cases [O] occurring within 15 km and 7 cases [+] occurring within 13 days) in each of the neighborhoods so that the number of cases occurring in the intersection of the two neighborhoods (⊕) equals exactly *k* (e.g., $k = 2$, the first and second nearest-neighbor in space-time). The radius is shown by the data, given a certain *n*. For a fixed chosen value of *k*, the value of *n* varies among cases and is found with a computer-intensive search algorithm. An example is shown of the two space-time nearest-neighbors of a given index case, by taking $n = 7$ at a radius of 15 km (in space), and 13 days (in time). The order is determined by increasing or decreasing the space-time intersection.

Results

During the 9-year surveillance period, 4,896 confirmed cases were noted. Of these, nine cases could not be used because of recording errors (Table 1). The dataset was made up of 250 different meningococcal serosubtypes, of which 42 were seen in 20 or more cases (4,189/4,887 = 86% of all strains), while 99 serosubtypes were only connected to one case (Appendix online, available from http://www.cdc.gov/ncidod/eid/vol10no9/03-0992_app.htm).

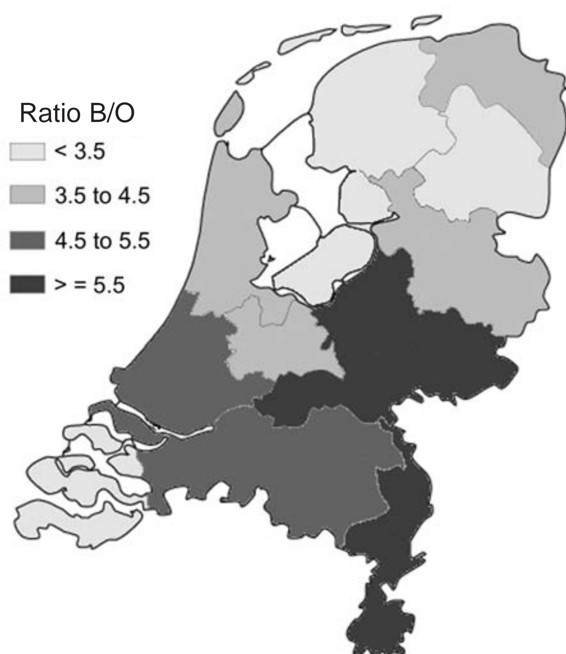

Figure 2. Distribution of the ratio of serogroup B to other serogroups (Ratio B/O) per province in the Netherlands (1993–2001).

The observed background value of cases in temporal and spatial proximity to an index case being of the same serosubtype is 12.0%. When random labeling was used, this percentage was 9.7%. We observed that 15.1% of the first nearest-neighbors were of the same serosubtype, an excess probability or secondary case percentage of 3.1% (CI 2.1%–4.1%). As most nearest-neighbors are coincidental, little difference was seen in the mean temporal and spatial distance between nearest-neighbors of the same serosubtype (6.1 km [range 0–44 km] and 13.2 days [range 0–63 days]) and those of different serosubtype (7.6 km [range 0–49 km] and 14.3 days [range 0–380 days]). The probability of the second, third, fourth, and fifth nearest-neighbors being of the same serosubtype did not differ significantly from background values (this difference was 0.6%, 0.3%, 0.8%, and 0.4%, respectively). For serogroup B, the excess probability was 3.1% (CI 2.0%–4.3%, n = 4,035) for the first nearest-neighbor. For serogroup C, the excess probability was 3.5% (CI 1.6%–5.3%; n = 728), and for serogroup W135 no excess probability was found (n = 59). Seven different serosubtypes, accounting for 14% (694/4,887) of all cases, showed significant excess probability (Table 2): B:1:P1.4 (12%), B:1:P1.16 (10%), B:4:P1.5 (20%), B:4:P1.10 (5%), B:nt:P1.nt (11%), B:15:P1.7 (11%), and B:15:P1.7,16 (7%).

The Municipal Public Health Services identified 40 field clusters involving 21 different serosubtypes: 11 primary school clusters (range 2–5 cases), 7 daycare center clusters (2–3 cases), 1 swimming pool cluster (4 cases), and 21 household clusters (2–3 cases). The cases all occurred within 21 days from the first case, and 78% (32/41) occurred within 8 days.

Six serosubtypes were identified by both methods as serosubtypes with clustering, 15 were identified only in field clusters, and 1 in statistical clustering only. Most field clusters consisted of only two cases (75%); this result is consistent with the results of our statistical approach.

Discussion

Our results suggest that in the context of current public health efforts, clustering of meningococcal disease is rare in the Netherlands and other Western countries. Our nearest-neighbor analysis provided a useful method of assessing the phenomenon of meningococcal clustering by taking random variance into account. Cases of the same serosubtype appeared beyond the expected background rate and were only seen in the first nearest-neighbor, which implies that only secondary cases occur in excess of chance (3.1%). Connections of more than two cases could not be demonstrated beyond chance. Throughout the year, invasive disease appears mostly as isolated cases. This limited clustering may reflect the positive effect of the prophylactic rifampicin policy; however, household field clusters are still reported, which possibly shows the constraints of this prevention policy. This paucity of real secondary cases is consistent with findings from other studies. A Belgian study found 4.4% secondary cases (range 2.0%–5.2%) in 1,913 cases of invasive meningococcal disease from 1971 through 1976 (7). In France, 37 (4.5%) co-primary and secondary cases were found in 814 reported cases from 1997 to 1988 (8). A Dutch study reported 1.4% co-primary and secondary cases among 507 cases from 1989 to 1990 (9). In England and Wales, 17 (0.5%) secondary cases were found among 3,256 cases from 1984 through 1987 (10). In a Danish study published in 2000, 1.2% secondary cases were observed in 172 cases of meningococcal disease (11).

Apart from proper prophylactic treatment, no additional measures could prevent further cases, since excess clustering only occurs in the first nearest-neighbors, while a cluster is only identified after at least two connected cases. The field cluster analysis confirms this assessment, since most new cases occur within a short period (78% within 8 days), occur geographically close to each other (patients are in the same household, daycare center, or primary school), and occur mostly in pairs (75%). These findings are consistent with observations in field cluster studies showing that secondary invasive disease most likely occurs nearby, within the next few days. In a Belgian study, 83% of 63 secondary cases occurred within 8 days of identifying the index case (7); in a French study, 31

Table 2. Clustering of meningococcal disease cases by serosubtype (serogroup, serotype, and subtype)[a,b]

Serosubtypes (phenotype)	Field clusters[c]	Cases/cluster	% excess probability	95% CI	n
B:1:P1.4	1 Primary school	3	**12.0**	4.2%–19.7%	87
B:1:P1.14	1 Swimming pool	4	7.2	NS (–8.0% to 22.4%)	25
B:1:P1.16	1 Primary school	3	**10.0**	2.4%–17.6%	92
	1 Daycare center	2			
	1 Household	2			
B:4:P1.2,5	1 Household	2	1.9	NS (–3.8% to 7.7%)	52
B:4:P1.4	2 Primary schools	3 and 5	2.0	NS (–0.7% to 4.6%)	1,376
	4 Primary schools	2			
	2 Daycare centers	2 and 3			
	6 Households	2			
B:4:P1.5	1 Daycare center	2	**20.0**	3.2%–36.9%	25
B:4:P1.7	NFC		6.4	NS (–2.7% to 15.5%)	47
B:4:P1.9	1 Primary school	3	7.2	NS (–8.0% to 22.4%)	36
B:4:P1.10	1 Household	2	**4.8**	0.1%–9.5%	205
B:4:P1.14	NFC		5.9	NS (–2.5% to 14.2%)	34
B:4:P1.15	NFC		4.0	NS (–0.4% to 8.4%)	129
B:4:P1.16	1 Household	2	2.2	NS (–2.4% to 6.8%)	63
B:4:P1.NT	1 Primary school	2	2.6	NS (–0.8% to 6.1%)	455
	1 Daycare center	2			
	1 Household	3			
B:NT:P1.14	2 Households	2 and 3	13.0	NS (–1.9% to 27.9%)	23
B:NT:P1.15	1 Household	2	7.2	NS (–1.7% to 16.0%)	39
B:NT:P1.16	1 Household	2	10.0	NS (–4.4% to 24.4%)	20
B:NT:P1.NT	1 Household	2	**10.7**	4.7%–16.8%	123
B:14:P1.4	1 Daycare center	2	0.9	NS (1.4% to 12.2%)	85
	1 Household	2			
B:15:P1.7	NFC		**11.3**	0.1%–22.6%	53
B:15:P1.7,16	1 Primary school	2	**6.8**	1.4%–12.2%	109
B:15:P1.9	1 Household	2	10	NS (–1.4% to 21.4%)	30
B:16:P1.14	1 Household	2	—[d]		10
B:16:P1.2,5	1 Daycare center	3	13.6	NS (–1.9% to 29.2%)	22
C:2a:P1.2,5	1 Household	2	–1.0	NS (–5.4% to 3.4%)	164
C:NT:P1.5	NFC		10	NS (–4.4% to 24.4%)	20
C:14:P1.12	1 Household	2	—[d]		2

[a]The following results are shown: serosubtypes with reported field clusters, serosubtypes with significant excess probability for clustering by nearest-neighbor analysis, and serosubtypes with nonsignificant excess probability of more than 3% (**bold** percentages significant).
[b]CI, confidence interval; NT, not typable; NS, not significant.
[c]NFC, no field cluster was reported for this serotype.
[d]Calculating excess probability not possible because n is too small.

(82%) of 38 secondary cases occurred within 8 days (8). Almost all (94%, 29/31) of the secondary cases occurred within 8 days in a study in the United States from 1980 to 1993 with eight school and university clusters (12). Five secondary cases occurred within 8 days in a school outbreak of six cases with serogroup B meningococcal disease in the United States (13).

Space-time clustering methods, e.g., those using the spatial scan-statistic (14–17), have been used for surveillance purposes with the objective of identifying outbreaks. However, to our knowledge, such methods have not been used to explore the existence of, and quantify, the phenomenon of clustering in a specific infectious disease. For this purpose the Ederer-Myers-Mantel procedure has been used (18,19); however, since this method requires separating space and time (e.g., into provinces and years), we considered it inappropriate for our purposes. Instead, we adapted the concept of nearest-neighborship to the two dimensions of space and time simultaneously (5,6).

Our study has several constraints. As many serosubtypes were rare, their individual clustering behavior could not be fully ascertained. We used place of residence as our geographic parameter, which could underestimate clustering, since transmission might occur at locations outside place of residence (such as work, school, and sport clubs). Most cases are found in children, who often spend time in daycare centers, schools, and other places outside the home. Since these places tend to be located in the same area as their homes, this factor likely did not affect our results. The extent of clustering was possibly overestimated because of imprecise geographic coordinates since our statistical method used the center of the municipality or postal code

area, but no more precise alternative is available. Since only phenotypic strain typing was conducted (serogroup, serotype, and subtype) and not the more sensitive porA-genotyping method that would have identified spurious clusters, background rates of clustering may have been overestimated. However, this method is unlikely to have affected the excess probability (3.1%) of clustering, since this rate is probably a result of direct transmission. Our method for calculating background value was chosen to be as realistic as possible; however, our results do not appear to be sensitive to the choice of 6th to 10th nearest-neighbors as a reference. For instance, results from 3rd to 10th nearest-neighbor or 7th to 10th nearest-neighbor, as a reference, were virtually identical.

We believe that our low observed incidence of secondary cases partly reflects the general inability to link cases connected by chains of transmission. As disease develops in only a few of the links in a chain of transmission, connected cases are unlikely to be still temporally and spatially close, which obviates detection. Not surprisingly, we found three times as many serosubtypes among reported field clusters (21 serosubtypes) than assessed with nearest-neighborship analysis (7 serosubtypes), which confirms that field clusters may be spurious. Although field clusters have low specificity, their sensitivity is presumably high. Genotyping can identify those clusters brought about by direct transmission; nevertheless, the value of cluster surveillance as a means of prevention is uncertain. Apparent clusters are not valuable to guide additional intervention efforts, since these would prevent few additional cases. Our method of space-time nearest-neighborship analysis provides a sensitive novel approach to the epidemiology of meningococcal disease and possibly even other infectious diseases.

Dr. Hoebe is a consultant on communicable disease control and a member of the National Working Party for Infectious Diseases, the Netherlands. His research interests focus on outbreak investigation.

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Methicillin-resistant *Staphylococcus aureus* in Europe, 1999–2002

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We explored the variation in proportions of methicillin-resistant *Staphylococcus aureus* (MRSA) between and within countries participating in the European Antimicrobial Resistance Surveillance System and temporal trends in its occurrence. This system collects routine antimicrobial susceptibility tests for *S. aureus*. We examined data collected from January 1999 through December 2002 (50,759 isolates from 495 hospitals in 26 countries). MRSA prevalence varied almost 100-fold, from <1% in northern Europe to >40% in southern and western Europe. MRSA proportions significantly increased in Belgium, Germany, Ireland, the Netherlands, and the United Kingdom, and decreased in Slovenia. Within countries, MRSA proportions varied between hospitals with highest variance in countries with a prevalence of 5% to 20%. The observed trends should stimulate initiatives to control MRSA at national, regional, and hospital levels. The large differences between hospitals indicate that efforts may be most effective at regional and hospital levels.

Staphylococcus aureus is an important cause of community- and hospital-acquired infections. Infections caused by methicillin- or oxacillin-resistant *S. aureus* (MRSA) are mainly nosocomial and are increasingly reported from many countries worldwide (1). As MRSA strains are frequently resistant to many different classes of antimicrobial drugs, second- and third-line antimicrobial resistance is a growing concern (2). Surveillance of MRSA provides relevant information on the extent of the MRSA epidemic, identifies priorities for infection control and the need for adjustments in antimicrobial drug policy, and guides intervention programs (3).

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In Europe, several surveillance systems collect data on MRSA (4,5). Most collect data from specific types of hospitals, for certain periods, or information related to specific antimicrobial susceptibility patterns. The only ongoing initiative that continuously monitors antimicrobial resistance in most European countries is the European Antimicrobial Surveillance System (EARSS), funded by Directorate General for Health and Consumer Protection of the European Commission. This network connects national surveillance systems and provides comparable and validated results of routine antimicrobial susceptibility tests (AST) following standardized protocols from a representative set of laboratories per country (6). Timely and detailed feedback is given through a freely accessible and interactive Web site (http:\\www.earss.rivm.nl). EARSS was established in 1998 and currently connects >600 laboratories in 28 countries, which serve >100 million people. Preliminary EARSS results showed considerable differences in the proportions of MRSA across Europe (7,8).

We report results of antimicrobial susceptibility testing of *S. aureus* blood isolates from 1999 to 2002 in Europe;

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these results show variation in the prevalence of MRSA, including variation in its proportions at the hospital level. To assess recent changes in the epidemiology of MRSA within countries, we also present country-specific temporal trends in the occurrence of MRSA.

Materials and Methods

Data Collection

Data (identification number of isolate, EARSS laboratory code, date and type of specimen, sex and age of patient, EARSS hospital code, hospital ward to which patient is admitted, result of *mecA* gene polymerase chain reaction [PCR], and susceptibility to several antimicrobial drugs, including oxacillin and vancomycin) are collected through national surveillance systems. AST results of every first *S. aureus* blood isolate per patient per quarter are submitted to the EARSS database by national data managers. After authorization by the national representatives by using standard feedback reports, national data are included in the EARSS database and become available on the Web site.

Susceptibility Testing

Antimicrobial susceptibility is tested according to a standardized protocol (5). Briefly, laboratories report oxacillin susceptibility, preferably determined by an oxacillin-screening plate or an oxacillin disk-diffusion test. To confirm methicillin resistance, the minimum inhibitory concentration (MIC) for oxacillin or the presence of *mecA* gene by PCR is determined. Reporting vancomycin MIC is recommended for MRSA isolates.

Interpretative AST results (i.e., sensitive [S], intermediate [I], and resistant [R], in accordance with defined guidelines) are accepted. Most (71%) of the laboratories have adopted the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS; www.nccls.org). Most guidelines agree that *S. aureus* isolates should be considered nonsusceptible (R) to oxacillin if the MIC is ≥4 mg/L. Lower MIC breakpoints (R if MIC ≥2 mg/L) are only suggested by the Deutsche Industrie-Norm (DIN) (www.din.de) and guidelines of the Swedish Reference Group for Antibiotics (SRGA) (www.srga.org).

Data Analysis

We rejected observations lacking mandatory information (i.e., laboratory code, date of specimen, either patient identification number or month and year of birth, pathogen code, antibiotic code, or oxacillin test result [S or R]); duplicate records and repeat isolates from the same patient were also rejected. Isolates with an interpretative AST result of "R" (resistant) to oxacillin or one of its equivalents (cloxacillin, dicloxacillin, and flucloxacillin) were defined as MRSA. Isolates with intermediate susceptibility were not counted as MRSA and were excluded from the analyses. MRSA proportions were calculated as the number of MRSA isolates divided by the total number of *S. aureus* isolates obtained from blood cultures.

For the current analysis, data collected from January 1999 through December 2002 were used. We included only information from hospitals with data for ≥ 20 isolates from countries reporting >100 isolates. To calculate time trends for analyses of variation between hospitals, we included only those hospitals that had participated in at least 3 consecutive years.

Univariate analyses were performed by using chi-square or *t* tests if appropriate. Country-specific trends in the occurrence of MRSA over time were analyzed by using a multivariate Poisson regression model adjusting for autocorrelation in hospitals (e.g., attributable to possible similarity in blood culturing and AST practice). We also compared countries with respect to variation between hospitals, expressed as the variance in hospital-specific MRSA proportions. To eliminate the natural dependency between variance and mean, the MRSA ratio was first transformed by power (Box-Cox) transformation according to the following formula: $T(k/n) = (k/n)^{\lambda}$, where T is the transformed MRSA ratio, k/n is the resistance rate (i.e., the number of resistant isolates divided by the total number of isolates), and λ was chosen in such a way that variance was independent of the mean, i.e., $\lambda = 0.397$. The variance was further adjusted by size (in terms of number of isolates reported) of individual hospitals. Country-specific variances were then graphically displayed and compared.

Results

From January 1999 through December 2002, EARSS received AST results of 53,264 *S. aureus* blood isolates from 27 countries (Norway does not report *S. aureus* data), including 628 laboratories serving 896 hospitals. Twenty-six countries reported AST results of >100 isolates. The current study included 50,759 isolates from 428 laboratories serving approximately 500 hospitals. Overall, 20% of these isolates were reported as methicillin resistant. A total of 295 hospitals (35,921 isolates, 19 countries) provided data for at least 3 consecutive years and were included in the time trend analyses. Table 1 describes the main characteristics of the data and the proportion of MRSA by country.

MRSA was more frequently isolated from men (21%) than from women (18%, $p < 0.001$). Patients with a blood culture positive for MRSA were older than patients with methicillin-susceptible *S. aureus* (MSSA) (mean age, 65.3 [SD 18.7] versus 58.6 [23.4], $p < 0.001$). The proportion of MRSA was highest among patients admitted to intensive care units (35%).

Table 1. Characteristics of EARSS database by countries[a,b]

Country (EARSS country code)	No. of hospitals[c]	Total no. of isolates	No. of MRSA isolates (%)	Period of participation
Austria (AT)	11	656	58 (8.8)	Jan 2000–Dec 2002
Belgium (BE)	36	2,953	696 (23.6)	Jul 1999–Dec 2002
Bulgaria (BG)	4	183	62 (33.9)	Jan 2000–Dec 2002
Croatia (HR)	6	341	125 (36.7)	Jul 2001–Dec 2002
Czech Republic (CZ)	35	2,426	142 (5.9)	Apr 2000–Dec 2002
Denmark (DK)	22	2,406	14 (0.6)	Jan 1999–Sept 2002
Estonia (EE)	3	112	1 (0.9)	Jan 2001–Dec 2002
Finland (FI)	17	1,990	19 (1.0)	Jan 1999–Dec 2002
France (FR)	24	3,376	1,117 (33.1)	Jan 2001–Dec 2002
Germany (DE)	25	3,757	600 (13.8)	Jan 1999–Dec 2002
Greece (GR)	19	1,126	500 (44.4)	Jan 1999–Dec 2001; Jul 2002–Dec 2002
Hungary (HU)	12	435	31 (7.1)	Jan 2001–Dec 2002
Iceland (IS)	1	184	1 (0.5)	Jan 1999–Dec 2002
Ireland (IE)	19	2,897	1,192 (41.2)	Jan 1999–Dec 2002
Israel (IL)	5	849	326 (38.4)	Jan 2001–Dec 2002
Italy (IT)	57	3,593	1,470 (40.9)	Jan 1999–Jun 2000; Apr 2001–Dec 2002
Luxemburg (LU)	4	214	41 (19.2)	Jan 1999–Dec 2002
Malta (MT)	1	240	105 (43.8)	Jan 2000–Dec 2002
Netherlands (NL)	45	5,359	30 (0.6)	Jan 1999–Dec 2002
Poland (PL)	8	238	42 (17.7)	Jan 2001–Dec 2002
Portugal (PT)	15	1,540	535 (34.7)	Jan 1999–Dec 2002
Slovakia (SK)	7	228	24 (10.5)	Jul 2001–Dec 2002
Slovenia (SI)	8	657	121 (18.4)	Jul 2000–Dec 2002
Spain (ES)	35	2,985	739 (24.8)	Jan 2000–Dec 2002
Sweden (SE)	54	6,071	48 (0.8)	Jan 1999–Dec 2002
United Kingdom (UK)	27	5,343	2,217 (41.5)	Jan 1999–Sept 2002
Total	500	50,759	10,256 (20.2)	

[a]EARSS, European Antibiotic Resistance Surveillance System; MRSA, methicillin-resistant *Staphylococcus aureus*.
[b]Only hospitals providing data of >20 isolates are included.
[c]According to EARSS hospital codes provided by the countries.

Geographic variation is displayed in Figure 1, which shows a north-south gradient, with the lowest MRSA prevalence in northern Europe and highest prevalence in southern Europe, Israel, the United Kingdom, and Ireland. MRSA proportions varied almost 100-fold, with the lowest proportion in Iceland (0.5%) and the highest proportion in Greece (44%, Table 1).

Statistical analyses of country-specific time trends by Poisson regression (Table 2) showed that increases in MRSA proportions were significant in Belgium (from 22% in 1999 to 27% in 2002), Ireland (39%–45%), Germany (9%–19%), the Netherlands (0.4%–1%) and the United Kingdom (31%–45%). The proportion of MRSA decreased significantly in Slovenia only, from 22% in 2000 to 15% in 2002. The model had difficulties in estimating changes in MRSA proportion in countries with low counts of MRSA isolates, which is reflected in the very wide confidence intervals for Iceland and Bulgaria (Table 2). Relatively large year-to-year fluctuations occurred in some countries (Bulgaria, Greece, Luxembourg, Malta, and Portugal); some of these countries (Bulgaria, Luxembourg, and Malta) had low isolate counts (Table 1). Figure 2 presents significant time trends by showing MRSA proportions per country per year for 1999 through 2002.

Figure 3A shows regional variation in MRSA proportions within countries. Particularly high variation was identified among hospitals in Belgium, the Czech Republic, Spain, Greece, Italy, Portugal, and the United Kingdom. After applying the power transformation, the remaining variation was highest in Germany (Figure 3B), with a variance after transform of 17%. Other countries with relatively high variation in MRSA proportions (variance after transform >15%) between hospitals were Poland, the Czech Republic, and Slovakia. The highest relative variation was found in countries with MRSA proportions from 5% to 20%, with the exception of Hungary and Slovenia. A relatively high variation between hospitals was also found in countries with MRSA proportions >25%. The lowest variation between hospitals was observed for Slovenia (variance after transform, 3%), and variation was also low in France (variance after transform, 5%).

Vancomycin resistance did not occur. Intermediate susceptibility of *S. aureus* (VISA) was only reported for five isolates from France in 2001.

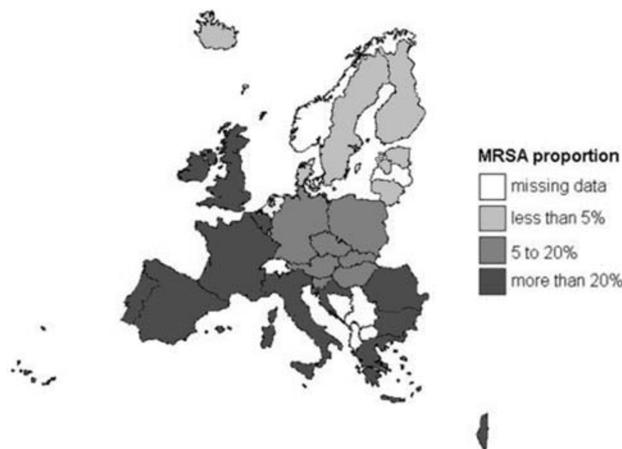

Figure 1. Geographic variation in proportions of methicillin-resistant *Staphylococcus aureus* (MRSA) (1999–2002).

Discussion

This is the first EARSS report on the prevalence of MRSA among blood isolates in 27 countries in the European region. We found that proportions of MRSA vary largely across Europe, with the highest proportions in southern and parts of western Europe and lowest proportions in northern Europe. MRSA proportions seem to be increasing in many countries. Significant increases were found for Belgium, Germany, the Netherlands, Ireland, and the United Kingdom, whereas the proportion of MRSA decreased in Slovenia. In all countries, variation between hospitals was observed. The variation between hospitals was highest in Germany and in most other countries with an MRSA prevalence of 5% to 20%. The lowest variation between hospitals was found in Slovenia.

Our results show the European situation with respect to the occurrence of MRSA in blood isolates and confirm other observations (9–11) on invasive isolates; they are also in accordance with findings of other studies with respect to demographic variables, such as sex, age, and patient ward (9,12). Although blood isolates represent the minority of clinically relevant samples, they are indicative of infection. Studies that report MRSA proportions from all sources usually include screening samples that are subject to bias because of differential screening practices. Considering hospital-acquired MRSA only seems to provide insight into the European MRSA epidemic, as the prevalence of community-acquired MRSA in Europe remains very low (0.03%–1.5%), even in countries with a high MRSA prevalence in hospitals (13–17). EARSS provides comparable data, annually validated through external quality assurance exercises, which have repeatedly confirmed a good-to-excellent concordance for identifying MRSA (18).

EARSS accepts susceptibility data according to clinical breakpoints (S, I, R) in agreement with international guidelines. Methicillin resistance is usually defined as having an MIC of ≥4 mg/L. Because of lower breakpoints (MIC ≥2 mg/L) defined by SRGA and DIN, this definition may

Table 2. Relative change in MRSA proportion per country per year and 95% confidence intervals as calculated from Poisson regression models [a,b,c]

Country	Reported % MRSA at start	Reported % MRSA in 2002	Relative change per year, ratio	95% CI of estimated change	p value
Austria	7.0[d]	7.6	0.80	0.48 – 1.34	0.39
Belgium	22.1	27.2	1.25	1.12 – 1.41	< 0.01
Bulgaria	35.1[d]	37.7	1.11	0.59 – 2.09	0.76
Czech Republic	4.5[d]	6.2	1.15	0.89 – 1.50	0.29
Denmark	0.3	1.0	1.64	0.97 – 2.75	0.06
Finland	1.5	0.8	0.69	0.43 – 1.11	0.13
Germany	9.4	19.2	1.72	1.54 – 1.93	< 0.01
Greece	37.0	48.6	1.23	0.89 – 1.71	0.21
Iceland	0.0	0.0	0.52	0.07 – 3.67	0.51
Ireland	39.4	45.0	1.36	1.17 – 1.58	< 0.01
Italy	35.2	40.0	1.11	0.94 – 1.30	0.23
Luxembourg	15.0	18.3	1.09	0.71 – 1.67	0.70
Malta	34.7[d]	42.5	1.58	0.92 – 2.74	0.10
Netherlands	0.4	1.0	1.62	1.01 – 2.58	0.04
Portugal	39.7	38.9	0.91	0.75 – 1.09	0.32
Slovenia	22.3[d]	14.7	0.69	0.51 – 0.93	0.02
Spain	28.4[d]	23.5	1.03	0.87 – 1.21	0.74
Sweden	1.1	0.7	0.95	0.73 – 1.23	0.68
United Kingdom	30.5	44.5	1.48	1.31 – 1.66	< 0.01

[a]CI, confidence interval; MRSA, methicillin-resistant *Staphylococcus aureus*.
[b]Adjusted for autocorrelation within hospitals and for variation in the number of isolates per quarter, including only the hospitals participating for at least 3 consecutive years and reporting data of > 20 isolates.
[c]The change estimated by the model does not necessarily correspond to the overall change that can be calculated from the second and third column, this is because some trends first show an increase, followed by a decrease, or vice versa.
[d]Data from year 2000 onwards.

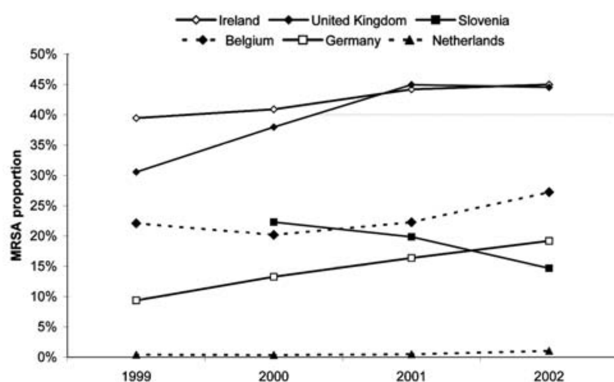

Figure 2. Statistically significant trends ($p < 0.05$) in methicillin-resistant *Staphylococcus aureus* (MRSA) proportions per year by country, 1999–2002, including hospitals participating for at least 3 consecutive years and reporting data of >20 isolates only.

have caused partial overestimation of MRSA proportions reported from Sweden (where SRGA is used in 100% of laboratories), and from Germany (where DIN is used in 59% of the participating laboratories) in comparison to other countries (19). However, most MRSA strains show high-level resistance to oxacillin, although low-level resistant strains are emerging (20). Moreover, such misclassification is unlikely to bias the country-specific temporal trends reported here. In all other countries, all laboratories agree on a single breakpoint (≥4 mg/L).

We used Poisson regression modeling adjusting for autocorrelation within hospitals to test for possible time trends in MRSA proportions. This model assumes that the epidemic runs according to an *S*-curve (21). The results of this analysis need to be interpreted with caution, as confidence intervals are wide, especially for countries with a low number of isolates. Year-to-year fluctuations found for some countries were probably not due to changes in the case-mix, as analyses were performed on data from a constant set of hospitals in each country, but were possibly caused by random variation of low numbers of isolates (Bulgaria, Luxembourg, Malta). Since the model estimates time trends over the 4-year observation period, it did not account for such fluctuations, which should be possible by autoregressive moving average (ARIMA) modeling (22). However, ARIMA modeling requires at least 60 data points, which cannot be provided at this stage.

The temporal increase we found for Germany is supported by a national surveillance study carried out at regular intervals, which reported an increase of MRSA from 2% in 1992 to 21% in 2001 (23,24). Our results for the United Kingdom show that the increase in MRSA proportions, reported from 1992 through 1998 (25,26), continued until 2001, and now appears to have leveled off. This

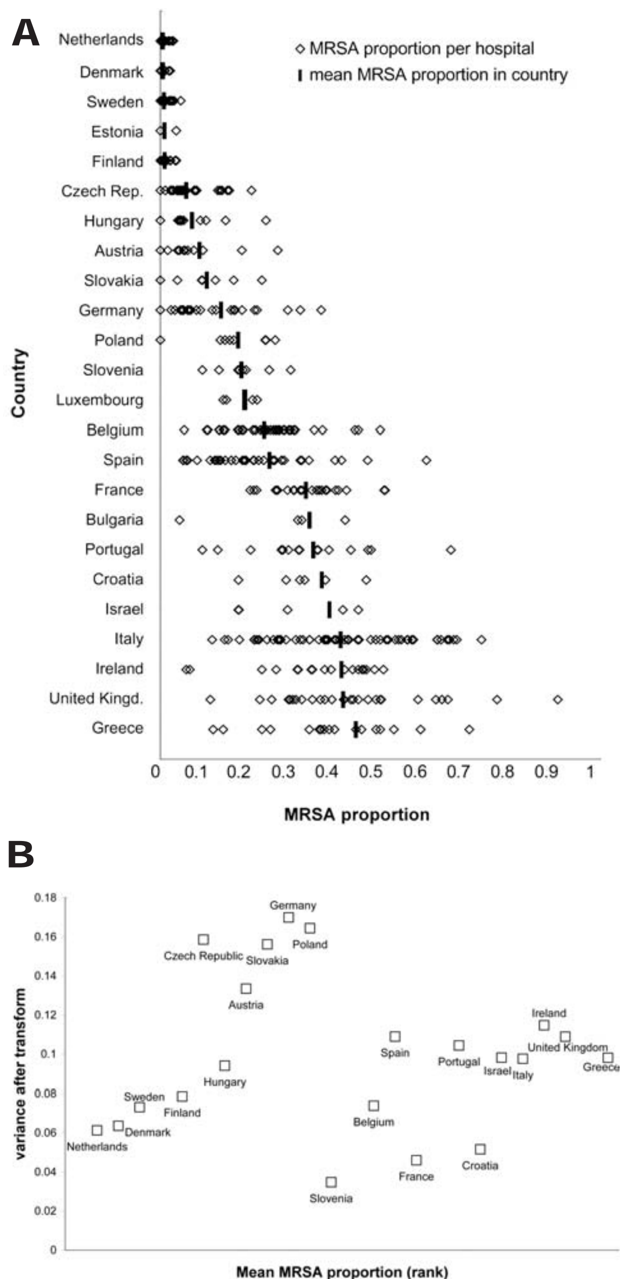

Figure 3. A) Variation in methicillin-resistant *Staphylococcus aureus* (MRSA) proportions between hospitals with AST results of >20 blood isolates, displayed by ranking of MRSA proportion (from lowest to highest). Only countries with more than one hospital are displayed. Hospital-specific proportions (open diamonds) are grouped per country. The solid vertical bars represent the mean MRSA proportion per country. B) Local variation, showing the power-transformed variance being independent of the mean MRSA proportion per country, displayed by ranking of MRSA proportion (from lowest to highest). Only hospitals reporting >20 isolates are included. Countries with less than five reporting hospitals are not shown (Iceland and Malta [1], Estonia [3], Bulgaria and Luxembourg [4]).

development in the MRSA epidemic reflects the curve of the number of hospitals affected by MRSA outbreaks over time, as predicted by Austin and Anderson (21). The same epidemic curve might apply to Ireland, although the stabilizing MRSA prevalence may also be the result of a nationwide infection control campaign (27). This Strategy for the Control of Antimicrobial Resistance in Ireland (SARI) follows a multidisciplinary approach, focusing on surveillance of antimicrobial resistance and use as well as infection control and stewardship of antibiotic use in the community and in hospitals. National MRSA guidelines are being updated, and the deficit in hospital staffing (laboratory surveillance scientists, infection control nurses, clinical microbiologists, and clinical pharmacists) is currently being addressed (28). In England, several recent initiatives have the goals of increasing awareness and encouraging efforts to control MRSA by individual hospitals. First, a mandatory surveillance program for MRSA bacteremia was launched, which included publication of MRSA diagnoses by named health trust (29,30). Second, a strategy was published to reduce healthcare-associated infection in England (31), which included guidelines for good hospital practice. The rise in MRSA prevalence in the Netherlands might be the result of the increase in heterogeneously resistant clones with low MICs for oxacillin (4–24 mg/L) (32). The effects of national infection control campaigns launched in Slovenia (J. Kolman, pers. comm.) may have had an impact. With the continuation of EARSS, we will be able to monitor any effect of such campaigns.

Variations in MRSA proportions between hospitals within the same country have been reported (9,33–35), but to our knowledge, this is the first attempt to quantify variation between hospitals at the national level in a European study. We showed that considerable variation in MRSA proportions exists not only between countries but also between hospitals within a country. Regional variation might be explained by different phenomena. The emergence of MRSA is largely due to dissemination of clonal strains, and temporary hospital outbreaks are typically due to clonal expansion (36). If stringent control measures are taken to prevent further MRSA transmission, MRSA prevalence might subsequently be reduced to sporadic levels (12). However, the effectiveness of MRSA control depends on several factors, such as the existence and correct application of hygiene protocols to prevent transmission (hand hygiene, isolation practices, cohorting), level of care needed by patients (indicating host susceptibility), and antimicrobial drug prescription policies (which would influence selective pressure), which might differ between hospitals in a country (37). As Kotilainen and colleagues showed, quick and adequate measures at the hospital level, as well as at the regional level, may be successful in containing the MRSA epidemic (38). Regional variation may also be explained by differences in diagnostic practice and culturing activity and random errors, which may artificially increase variation (39). Also, a differential case-mix attributable to differences in the level of care provided per hospital and differential referral practice may confound our estimates (9,35,40). However, unusually high variation in MRSA proportions between hospitals seems to occur most often in countries experiencing a current surge of MRSA. In support of this hypothesis, in general, MRSA proportions varied most in countries with increasing and intermediate (5%–20%) MRSA prevalence. These countries might have changed from equilibrium with adequate control and elimination of sporadic MRSA and might be on the verge of becoming MRSA-endemic. This stage may be characterized by abandoning strict search-and-control strategies and adopting more flexible approaches, as happened in England when MRSA prevalence was increasing in the 1990s (41,42). However, MRSA proportions were not increasing in all these countries, and variation in prevalence between hospitals was also high in countries with a high overall MRSA prevalence (>25%) (37). In contrast, in Slovenia, where MRSA proportions have decreased recently, variation between hospitals was low. Thus, the national campaign on infection control might have decreased not only MRSA prevalence but also the variation in MRSA proportions between hospitals.

Our database did not show vancomycin resistance; a few VISA isolates were reported from France only. This finding might be explained by the fact that EARSS collects routine data, whereas VISA will only be detected in specialized laboratories. Moreover, the clinical and epidemiologic importance of (heterogeneous) VISA remains to be clarified.

EARSS results show that MRSA proportions increased in several countries. Variation in MRSA proportions exists at international and at national levels, and regional variation seems to be highest in countries with intermediate MRSA proportions (5%–20%). Although the reasons for this phenomenon are unknown, high variation may occur in countries where the epidemiology of MRSA is in a transition period (e.g., Germany). Also in countries with a high MRSA proportion, between-hospital variation remains considerable. The large differences between hospitals indicate that initiatives may be most effective when undertaken at the local or regional level (38). To combat the MRSA epidemic, public health researchers and all health professionals must understand the role of hospital hygiene protocols and of antimicrobial drug policies, as well as mechanisms of regional spread of MRSA throughout hospitals. Studies that link information on MRSA guidelines, antimicrobial policies, and prescriptions with resistance rates at the level of the hospital, region, or both, may increase our understanding of the nature of the MRSA epidemic (43).

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Outbreak of Hantavirus Pulmonary Syndrome, Los Santos, Panama, 1999–2000

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An outbreak of hantavirus pulmonary syndrome occurred in the province of Los Santos, Panama, in late 1999 and early 2000. Eleven cases were identified; 9 were confirmed by serology. Three cases were fatal; however, no confirmed case-patient died. Case-neighborhood serologic surveys resulted in an overall hantavirus antibody prevalence of 13% among household and neighborhood members from the outbreak foci. Epidemiologic investigations did not suggest person-to-person transmission of hantavirus infection. By use of Sin Nombre virus antigen, hantavirus antibodies were detected in *Oligoryzomys fulvescens* and *Zygodontomys brevicauda cherriei*. This outbreak resulted in the first documented cases of human hantavirus infections in Central America.

Hantavirus pulmonary syndrome (HPS) is an infectious disease typically characterized by fever, myalgia, and headache and followed by dyspnea, noncardiogenic pulmonary edema, hypotension, and shock (1,2). Common laboratory findings include elevated hematocrit, leukocytosis with the presence of immunoblasts, and thrombocytopenia (3,4). The case-fatality rate can be as high as 52% (5). The etiologic agent of HPS is any one of several hantaviruses carried by rodent hosts belonging to the family *Muridae*, subfamily Sigmodontinae (6). Hantaviruses are most often transmitted to humans through the inhalation of infectious rodent feces, urine, or saliva. However, strain-specific virus transmission may occur from person to person (7–9).

HPS was first recognized in 1993 during an outbreak of severe respiratory disease in the Four Corners Region of the United States (10,11). Since then, 363 cases of HPS have been confirmed in the United States (12). Sin Nombre virus (SNV) was the first HPS-causing pathogen identified; its primary rodent reservoir host is the deer mouse, *Peromyscus maniculatus* (13,14). However, four other hantaviruses, Bayou virus, Black Creek Canal virus, New York virus, and Monongahela virus, each with a different rodent reservoir, have been characterized in the United States and associated with HPS (6,15–21).

Since 1993, HPS has also been reported and confirmed in six countries in South America—Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay (6,9,22–30). Several distinct hantaviruses have been associated with HPS, including Andes virus (Argentina, Bolivia, Chile, and Uruguay), Bermejo virus (Bolivia), Juquitiba virus (Brazil), Laguna Negra virus (Bolivia, Paraguay), Lechiguanas virus (Argentina), and Oran virus (Argentina) (22,23,25,28,30,31).

Before 2000, no human hantavirus infections had been reported in Central America. However, in February 2000, health officials in Panama reported a cluster of acute respiratory illnesses in residents of the district of Las Tablas in Los Santos Province from late December 1999 to February 2000 (32). Human illness was characterized by a febrile prodrome with rapid progression to moderate-to-severe respiratory distress. Serum specimens from three of four patients had immunoglobulin (Ig) M and IgG antibodies to SNV. In February and March 2000, an outbreak investiga-

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tion by the Panamanian Ministry of Health, Gorgas Memorial Institute for Health Studies (Panama City), the U.S. Centers for Disease Control and Prevention (Atlanta, GA), and the Pan American Health Organization was conducted in collaboration with local medical and public health officials. Sequence analysis of virus genome from human serum samples and rodent tissue led to the identification of a novel hantavirus, Choclo virus, as the cause of HPS in this outbreak (33). This report summarizes the clinical, epidemiologic, and environmental findings of the investigation.

Materials and Methods

Case Definition

A suspected case of HPS was defined as fever (temperature ≥38.3°C) and unexplained acute respiratory distress requiring supplemental oxygen, with radiographic evidence of acute respiratory distress syndrome or bilateral interstitial pulmonary infiltrates (34). A suspected case was also defined as an unexplained respiratory illness resulting in death, with a postmortem examination indicating noncardiogenic pulmonary edema without identifiable cause (34). A confirmed case of HPS was defined as a clinically compatible illness plus the presence of one of the following; 1) hantavirus-specific IgM antibodies in acute-phase serum, 2) hantavirus-specific nucleic acid sequences by reverse transcriptase polymerase chain reaction (RT-PCR), or 3) hantavirus-specific antigen by immunohistochemistry (34).

Case Finding and Characterization

At visits to two hospitals in Panama City and one in the Las Tablas District that admitted patients with suspected HPS, hospitalized patients were examined and interviewed when possible, and medical charts were reviewed. To identify past HPS patients, retrospective chart reviews were conducted on admissions dating back to August 1999 at these hospitals. In addition, medical records of any previous or current suspected HPS patients admitted to other district hospitals in the Las Tablas Province were obtained and reviewed. Clinical case information was collected on a standard abstraction form.

To monitor the spread of disease in Las Tablas and other areas of Los Santos Province, an outbreak communications center was established at the Ministry of Health in Panama City and staffed by physicians, public health officials, and health educators. Operations of this center included 1) passive surveillance for suspected cases of HPS, 2) a public hotline that addressed symptoms and signs of HPS and methods of prevention, and 3) nationwide distribution of HPS educational materials. Staff physicians also called hospitals throughout Panama to promote awareness of HPS among medical providers.

Community Surveys

Surveys for hantavirus antibodies were conducted on March 5, 2000, in six neighborhoods in the Las Tablas District and one in the Guarare District in which confirmed HPS patients resided. The primary objectives of the surveys were to determine the prevalence of hantavirus infection within households and neighborhoods of case-patients and the frequency of mild and asymptomatic infection.

Approximately 10–15 households, including the case-patient household, were sampled in each of the seven neighborhoods. Teams composed of public health officials, nurses, and phlebotomists visited each neighborhood. The day before the survey, pamphlets explaining the survey in Spanish were given to each household that had agreed to participate. All participants were administered a standard questionnaire that asked about demographic characteristics, illness history, and rodent exposure. Team members were familiarized with the questionnaire before the survey started. A 10-mL blood specimen (3–5 mL for children) for hantavirus serologic testing was collected from each interviewed participant.

Hospital Survey

A hospital survey was conducted March 9–10, 2000, among healthcare workers who cared for confirmed HPS patients at a major hospital in Panama City. This hospital received most of its cases from Las Tablas. The objectives of the survey were to 1) serologically determine exposure to hantavirus among doctors, nurses, respiratory therapists, physiotherapists, and nurses' aides who provided direct care to confirmed HPS patients and 2) assess whether person-to-person transmission occurred. Medical staff who provided direct medical care (i.e., ≤1 m from the patient) to infected patients in the emergency room and medical intensive care unit were compared with those in the coronary or neurologic intensive care units who had no exposure to case-patients. A standard self-administered questionnaire was used to inquire about timing and amount of exposures, specific types of exposures, and precautionary measures taken. A 10-mL blood specimen for hantavirus serologic testing was collected from each surveyed participant.

Rodent Investigation

Small mammals were sampled to determine potential hantavirus reservoir hosts. Primary sampling methods were trapping and collecting small mammals around households of confirmed and suspected HPS patients and from two uninhabited locations in Los Santos. The habitat of each trapping area was described. Small mammals were initially identified in the field on the basis of external characteristics and standard keys for the region. Definitive identification that used cranial characteristics was per-

formed at the Museum of Southwest Biology, University of New Mexico, where voucher specimens from these small mammals are currently archived. Liver, kidney, spleen, lung, heart, and a whole blood sample were collected from each trapped rodent for diagnostic testing, and carcasses were preserved for rodent speciation. Trapping and sampling were performed according to established safety protocols (35). Panamanian team members were trained to trap and observe safety precautions when handling potentially infectious rodents.

Diagnostic Testing

All available serum specimens from suspected HPS patients were tested for IgM antibodies by using an IgM-capture format and inactivated SNV antigen and for IgG antibodies by an indirect enzyme-linked immunosorbent assay (ELISA) as previously described (11). Serum samples from survey participants, rodents, and other small mammals were tested for IgG antibodies by using SNV antigens. IgG-positive survey participants were also tested for IgM antibodies by use of SNV antigens. Positive findings with SNV antigens in the IgG- or IgM-capture ELISAs indicate infections with New World hantaviruses rather than with SNV specifically.

Results

Characteristics of HPS Patients

Eleven case-patients with suspected HPS were identified and reported to the Panamanian Ministry of Health from December 25, 1999, to February 29, 2000 (Figure 1). Three patients (25%) died; neither serum nor tissue samples were available for diagnostic testing from these three patients. The remaining eight cases were confirmed by the presence of IgM against hantavirus in at least two separate serum specimens from patients; all eight also had IgG antibodies, with five demonstrating IgG seroconversion after the first specimen. One additional patient, whose onset of illness was August 24, 1999, was identified retrospectively. Although acute-phase clinical specimens were not available for testing, review of medical records indicated that her illness was clinically compatible with HPS, and her IgG titer in February 2000 was elevated (6,400). She was subsequently confirmed as the ninth HPS patient.

The median age of the 12 patients was 42 years (range 26–58 years); seven (58%) were women. The primary occupation of patients was as follows: secretary (2), housewife (2), truck driver/transporter (2), electrician (1), field pump worker (1), seamstress (1), teacher (1), security guard (1), and unidentified (1).

All cases occurred in the Las Tablas, Guarare, and Tonosi Districts of Los Santos Province. In Las Tablas and Guarare, eight towns had at least one patient with suspected HPS, and seven had one or more confirmed patients (Table 1). Two cases, one suspected and one confirmed, were identified in Tonosi. No clustering of cases occurred at the household level.

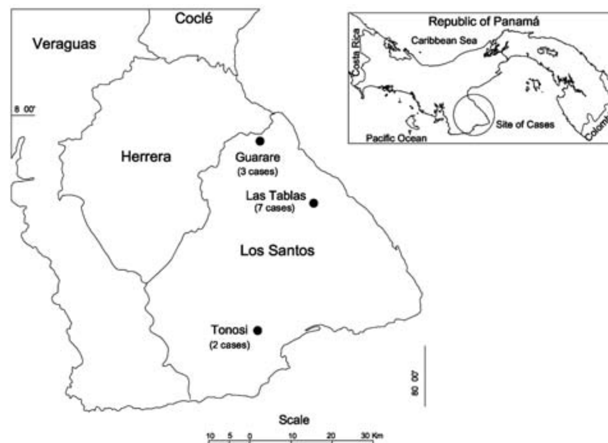

Figure 1. Location of districts in Los Santos Province, Republic of Panama, in which hantavirus cases occurred in 1999 and 2000.

Two patients denied handling or cleaning up rodent excreta before their illnesses, while a third admitted frequently killing and handling rodents. In interviews, family members of all case-patients from Las Tablas District noted marked increases in the number of rodents in and around the home from November 1999 to February 2000.

Clinical Description of Confirmed HPS Patients

The spectrum of preadmission symptoms of the nine confirmed patients is documented in Table 2. The mean temperature on admission was 38.0°C (n = 6, range 37.3°C–39.6°C). The median systolic and diastolic blood pressure values were 100 mm Hg (n = 9, range 80–130) and 63 mm Hg (n = 9, range 50–80), respectively. The median pulse rate was 102/min (n = 8, range 60–172). The median respiratory rate was 23/min (n = 8, range 14–36). No patient was unconscious or cyanotic on admission. A

Table 1. Geographic distribution of suspected and confirmed HPS cases, Los Santos Province, Panama, August 1999–February 2000[a]

Town	District	No. confirmed cases	No. suspected cases
El Cocal	Las Tablas	1	0
La Tronosa	Las Tablas	0	1
Las Tablas	Las Tablas	1	0
Los Angeles	Las Tablas	1	0
San Jose	Las Tablas	2	0
Santo Domingo	Las Tablas	1	0
Bella Vista	Guarare	1	0
La Enea	Guarare	1	1
Las Llanas	Tonosi	1	1

[a]HPS, hantavirus pulmonary syndrome.

Table 2. Symptoms on admission in nine patients with laboratory-confirmed hantavirus pulmonary syndrome, Los Santos Province, Panama, August 1999–February 2000

Symptom	No. (%)
Fever	9 (100)
Dyspnea	8 (89)
Myalgia	8 (89)
Cough	7 (78)
Malaise	6 (67)
Vomiting	5 (56)
Nausea	4 (44)
Headache	4 (44)
Arthralgia	4 (44)
Weakness	4 (44)
Lower back pain	3 (33)
Abdominal pain	2 (22)
Chest pain	2 (22)
Dizziness	2 (22)
Diarrhea	1 (11)
Dysuria	1 (11)
Lower extremity edema	1 (11)

temperature of ≥38.5°C was documented in all patients at some time during hospitalization. Seven of nine patients were hypotensive during their illness (median lowest systolic blood pressure = 90, range 50–100), and three required inotropic therapy.

Laboratory values at admission and during hospitalization are listed in Table 3. Eight of nine patients were thrombocytopenic either on admission or at some time during hospitalization. Five of nine had hematuria (urine erythroctyes >10 per high power field, while four of nine had moderate proteinuria (≤2+ on urine dipstick). Evidence of renal function abnormalities was present in three of nine patients whose serum creatinine levels were >2.0 mg/dL.

Three of nine patients had aspartate aminotransferase >500 U/L, alanine aminotransferase >300 U/L, total bilirubin >1.5 mg/dL, and direct bilirubin >1.0 mg/dL. Two patients had clinical evidence of liver disease—one with hepatomegaly and the other with scleral icterus. Hematemesis, melena, and coagulopathy (prothrombin time = 22 s, partial thromboplastin time = 167 s, fibrinogen = 161 mg/dL) also developed in one patient. Two of the three patients also had elevated serum amylase values (294 and 437 U/dL).

Seven of nine patients had chest x-ray examinations on admission. All had evidence of bilateral infiltrates, and one of seven had a radiographic pattern suggestive of pulmonary edema. Two patients who did not have chest x-ray examinations on admission subsequently were found to have bilateral interstitial infiltrates radiographically. Pulmonary edema of varying severity developed in four patients during the course of their illnesses; pleural effusions also occurred in three of these patients, and they were intubated within 24 to 72 hours after admission.

Community Survey

Interviews and serum specimens were obtained from 311 (83%) of 376 residents of seven different neighborhoods of Las Tablas and Guarare Districts in which confirmed HPS patients were identified. These 311 survey participants represented 119 households. A minimum of one blood specimen was obtained from each household. Forty (13%) of 311 survey participants had IgG against

Table 3. Laboratory values of confirmed HPS patients on admission and during hospitalization[a]

Test	No. of cases	Median value (admission)	Range	No. of cases	Median maximum value (admission + hospitalization)	Range	Median minimum value (admission + hospitalization)	Range
Leukocyte count (x1,000/mm^3)	7	6.4	3.6–14.1	9	11.4	5.7–28.1		
Hematocrit (%)	7	39.7	35.1–44.2	9	41.0	31.4–52.4		
Platelet (x1,000/mm^3)	7	84	47–186	9			60	26–429
BUN (mg/dL)	2	7	5–9	8	26	1–56		
Creatinine (mg/dL)	3	0.8	0.6–1.0	8	2.2	1.0–3.3		
CPK (IU/L)				6	240	38–12,840		
LDH (U/L)	1	219	219[b]	7	1028	32–3,775		
Albumin (g/dL)				7			2.5	1.2–3.2
AST (U/L)	2	42	40–43	7	223	46–983		
ALT (U/L)	2	33	31–35	7	168	32–570		
Bilirubin, total (mg/dL)				7	1.1	0.8–8.0		
Bilirubin, direct (mg/dL)				7	0.6	0.1–6.5		
Amylase (U/L)				6	74	38–437		
PT (s)	1	11.7	11.7–11.7	4	13.7	11.3–22.0		
PTT (s)	1	31.2	31.2[b]	4	28	27–167		
Urine leukocyte count (/hpf)	5	9	6–35	9	8	1–35		
Urine erythrocyte count (/hpf)	4	12	4–25	9	10	1–35		

[a]HPS, hantavirus pulmonary syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CPK, creatinine phosphokinase; LDH, lactate dehydrogenase; PT, prothrombin time; PTT, partial thromboplastin time; hpf, high power field.
[b]There was only one case for this laboratory category.

Table 4. Hantavirus IgG antibody-positive findings of survey participants and households by neighborhood, town, and district of confirmed HPS patients, Los Santos Province[a]

Neighborhood (case no.)	Town	District	No. of persons tested	No. (%) persons antibody positive	No. of households tested	No. (%) households antibody positive
2	Los Angeles	Las Tablas	45	4 (8.9)	18	4 (22.2)
4	San Jose	Las Tablas	34	5 (14.7)	11	3 (27.3)
7	Bella Vista	Guarare	50	3 (6.0)	24	2 (8.3)
8	San Jose	Las Tablas	48	15 (31.3)	15	11 (73.3)
9	Santo Domingo	Las Tablas	43	6 (14.0)	15	4 (26.7)
11	Las Tablas	Las Tablas	41	4 (9.8)	15	4 (26.7)
12	El Cocal	Las Tablas	50	3 (6.0)	21	3 (14.3)
Total			311	40 (12.9)	119	31 (26.1)

[a]IgG, immunoglobulin G; HPS, hantavirus pulmonary syndrome.

hantavirus. By sex, 25 (14%) of 178 females and 15 (11%) of 133 males had IgG antibodies. The median age of the 40 antibody-positive participants was 31.5 years (range 1–79). Age group prevalence estimates ranged from 9% (5/53, >61years of age) to 21% (6/28, 51–60 years of age). Of 47 children, 5 (11%) ≤10 years of age had IgG antibodies. Antibody prevalences among patients' neighborhoods ranged from 6% to 31% (Table 4). Two of 10 household members of confirmed patients had IgG antibodies. Six (15%) of the 40 infected participants had visited a confirmed HPS patient.

Of 119 households surveyed, 31 (26%) had at least one member who had IgG against hantavirus (Table 4). Eight (8%) of 96 households had two or more antibody-positive members, while 1 (2%) of 54 households had three or more antibody-positive members. Prevalence of antibody-positive households (i.e., one or more antibody-positive members) per neighborhood ranged from 8% to 73%.

Among the 40 antibody-positive participants, occupations of those with the highest antibody prevalences were students (14%), secretaries (13%), agricultural workers (13%), livestock or vegetable farmers (12%), and housewives (11%). Among the 40 infected participants, 33 (82.5%) touched rodents, 31 (77.5%) cleaned up rodent droppings (e.g., sweeping, mopping), and 29 (72.5%) killed rodents after December 1, 1999. Fifteen (37.5%) noted increased numbers of peridomestic rodents compared with previous years.

Only five (12.5%) of 40 antibody-positive participants recalled fever or myalgia since December 1, 1999. In contrast, the most common symptoms reported were upper respiratory in nature, such as rhinorrhea (45%), sore throat (35%), and cough (22%). Moreover, of two participants who had both IgM and IgG antibodies, one experienced only a cough without fever, while the other was asymptomatic.

Hospital Serosurvey

Questionnaires and serum samples were obtained from 38 directly exposed and 39 unexposed healthcare workers. No IgM antibodies were present in the 77 workers. Only one of 38 exposed workers had IgG antibodies. This person, a medical resident, directly cared for five confirmed HPS patients (each healthcare worker cared for an average of 4 patients, range 1–5) for a total of approximately 15 patient-care days (group mean 43, range 1–70). His first exposure occurred in late December 1999. While caring for patients, he wore gloves, gown, and mask most of the time and was not directly exposed to respiratory secretions. He denied any travel to Los Santos Province after December 1999 and had no history of exposure to rodents. However, he was uncertain about whether he had visited Los Santos Province before December 1999. He denied having any febrile illness after late December 1999. Of the remaining 37 healthcare workers who cared for HPS patients, four (11%) were directly exposed to respiratory secretions in the eye, nose, or mouth; 26 (70%) wore gloves, gowns, and masks most of the time.

One unexposed worker, an operating room assistant, also had IgG against hantavirus. She denied travel to Los Santos Province, exposure to rodents, or febrile illness after December 1999. However, history of travel to Los Santos Province before December 1999 was again uncertain.

Rodent Investigation

Rodent traps were set at 13 sites, 10 of which were homes and immediate surroundings of patients with confirmed and suspected HPS. One of the three remaining sites was the home of a patient previously suspected to have HPS who did not have antibodies to hantavirus. The other two locations were a rural agricultural area in the Pocri District of Los Santos Province and a late secondary forest area (two subsites) near the town of Portabelo, Montijo District, Veraguas Province. In all, 120 rodents representing nine species and seven opossums (two species) were captured (Table 5). Of the 120 rodents, 52 (43%) were caught from the 10 patient household areas. A trap success of approximately 5% was achieved at these homes. Only one of the six antibody-positive rodents (*Oligoryzomy fulvescens*) was captured at the household of

Table 5. List of species of rodents and opossums captured at 13 trapping sites in Los Santos and Veraguas Provinces, Panama, February–March 2000, and antibody results

Rodent species (common name)	No. captured	No. hantavirus antibody-positive
Zygodontomys brevicauda cherriei (cane rat)	50	4
Sigmodon hispidus (hispid cotton rat)	20	0
Oligoryzomys fulvescens (fulvous pigmy rice rat)	15	2
Oryzomys couesi (Coues's rice rat)	14	0
Mus musculus (house mouse)	9	0
Liomys adspersus (Panamanian spiny pocket mouse)	8	0
Oryzomys concolor (colored rice rat)	2	0
Proechimys semispinosus (silky spiny rat)	1	0
Rattus rattus (black rat)	1	0
Marmosa mexicana. (Mexican mouse opossum)	4	0
Marmosa robinsoni (Robinson's mouse opossum)	3	0

a confirmed HPS case-patient (Las Tablas town); five were captured in the Pocri District.

Discussion

We have documented the first human cases of hantavirus infection in Central America. A novel Panamanian hantavirus, Choclo virus, was subsequently characterized and is thought to be responsible for HPS during the outbreak (33). Virus genetic sequences of Choclo virus from case-patients were identical to those from *O. fulvescens* (33).

The clinical spectrum of pulmonary disease among HPS patients in Panama varied widely from severe disease requiring intubation and cardiovascular support to mild pulmonary involvement with a benign clinical course. Extrapulmonary manifestations, such as hepatobiliary disease, hemorrhage, and central nervous system sequelae, were also present. The case-fatality rate (0% among 9 confirmed cases; 25% among 12 total suspected and confirmed cases) was noticeably lower than that described in Paraguay (12%), Chile (54%), and the United States (52%) in confirmed cases (5,9,29).

We found an antibody prevalence of 13% among household and neighborhood members of all ages from the outbreak foci. This figure is comparable to that found in Paraguay, but higher than in Chile and the United States (9,29,36). The percentages of hantavirus antibody-positive persons and households were particularly high in San Jose, the only town with two confirmed case-patients. Clustering of cases of HPS was not observed, and household clustering of antibody-positive persons was infrequent. Only 13% of hantavirus antibody-positive persons had a febrile illness after early December 1999, and none had an illness compatible with HPS, which suggests that mild infections occurred, as documented with other hantaviruses in the United States and Chile (9,36–38).

Person-to-person transmission of hantavirus infection was not demonstrated during the outbreak. Infrequent clustering of antibody-positive serosurvey participants by household probably reflected common exposures to infected rodent excreta peridomestically. Furthermore, only 1 of 38 medical care workers who cared for HPS patients had IgG antibodies, while none had IgM antibodies. Similarly, 1 of 39 workers who did not care for HPS patients had IgG antibodies. Thus far, person-to-person transmission has only been suggested during outbreaks caused by Andes virus in Argentina and Chile (7–9).

Climate data from Los Santos Province clearly demonstrated a two- to threefold increase in rainfall in September and October 1999 when compared to similar periods in the previous 4 years (Figure 2). Such atypical rainfall patterns may have led to increases in rodent populations, which led to more frequent contact between infected rodents and humans and subsequent human infection. Many residents of patient-neighborhoods of Las Tablas reported an unusually large number of rodents from December 1999 through January 2000. Similar patterns of environmental change followed by outbreaks of human disease have been observed in the United States and South America (29,39,40).

Ecologic observations in Los Santos provided an impression of a dry, open, and deforested region. Long-term commercial logging, agriculture (primarily corn and sugarcane), and animal husbandry practices (mainly cattle and horses) have contributed to the deforestation process. The rodent species most closely associated with corn and sugarcane fields, *Zygodontomys brevicauda cherriei*, is a

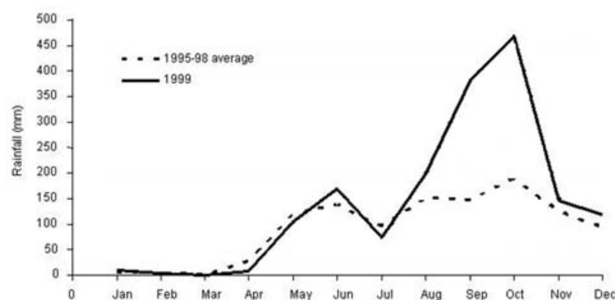

Figure 2. Comparison of monthly rainfall in 1999 with the average monthly rainfall from 1995 to 1998, Los Santos Province, Panama.

likely reservoir host for a new hantavirus, Calabazo virus (33). *O. fulvescens*, the likely reservoir for Choclo virus, was found in grass of varying heights near human habitation and in cattle and horse pastures. Given the observed habitat associations of these two species, agriculture and animal husbandry practices in the Los Santos regions may have had a positive effect on populations of rodents associated with hantaviruses and may continue to augment the risk for HPS as the human population increases.

The social and economic impact of this outbreak was substantial. The cancellation of Carnival, one of Panama's most celebrated festivals, had financial effects on residents of Las Tablas. Nevertheless, the public health impact of holding Carnival could also have been substantial, given the potential for rodent exposure among thousands of visitors and participants.

In conclusion, this outbreak resulted in the first documented cases of human hantavirus infections in Central America. Although cases were not reported from other districts of Los Santos or other provinces of Panama during the investigation, surveillance for HPS nationwide should continue, as serologic testing capabilities have since been implemented in Panama. More extensive sampling of rodent populations would help identify other areas in Panama with large numbers of *O. fulvescens* that could place residents at risk for Choclo virus infection. Longitudinal studies of rodents, particularly those species implicated as reservoir hosts, will be necessary to monitor the fluctuation and distribution of rodent population numbers over time and their correlation with human infection (41). Educational campaigns promoting risk reduction, such as proper clean-up of rodent excrement, sealing homes against the entry of rodents, and other rodent-proofing techniques, should continue as additional cases of HPS are reported in Panama.

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Detection of *Anaplasma phagocytophilum* DNA in *Ixodes* Ticks (Acari: *Ixodidae*) from Madeira Island and Setúbal District, Mainland Portugal

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A total of 278 *Ixodes* ticks, collected from Madeira Island and Setúbal District, mainland Portugal, were examined by polymerase chain reaction (PCR) for the presence of *Anaplasma phagocytophilum*. Six (4%) of 142 *Ixodes ricinus* nymphs collected in Madeira Island and 1 nymph and 1 male (2%) of 93 *I. ventalloi* collected in Setúbal District tested positive for *A. phagocytophilum msp*2 genes or *rrs*. Infection was not detected among 43 *I. ricinus* on mainland Portugal. All PCR products were confirmed by nucleotide sequencing to be identical or to be most closely related to *A. phagocytophilum*. To our knowledge, this is the first evidence of *A. phagocytophilum* in ticks from Setúbal District, mainland Portugal, and the first documentation of *Anaplasma* infection in *I. ventalloi*. Moreover, these findings confirm the persistence of *A. phagocytophilum* in Madeira Island's *I. ricinus*.

Anaplasma phagocytophilum (formerly *Ehrlichia phagocytophila*, *E. equi*, and the human granulocytic ehrlichiosis agent [HGE agent] [1]) is well established as a worldwide tickborne agent of veterinary importance and is considered an emerging human pathogen. The initial reports of human disease caused by *A. phagocytophilum*, now called human granulocytic anaplasmosis, came from Minnesota and Wisconsin in 1994 (2,3). Human granulocytic anaplasmosis is an acute, nonspecific febrile illness characterized by headache, myalgias, malaise, and hematologic abnormalities, such as thrombocytopenia and leukopenia as well as elevated levels of hepatic transaminases (4). Since that first report, an increasing number of cases have been described, mostly in the upper Midwest and in the Northeast regions of the United States (5). Three years later, in 1997, acute cases of this disease were also described in Europe (6,7). Several serologic and polymerase chain reaction (PCR)-based studies described the wide distribution of *A. phagocytophilum* across Europe and in some parts of the Middle East and Asia (8–10). Nevertheless, confirmed cases of human granulocytic anaplasmosis are rare; most European cases are described in Slovenia (11), with only a few reports from other European countries (12) and China (13).

The ecology of *A. phagocytophilum* is still being defined, but the agent is thought to be maintained in nature in a tick-rodent cycle, similar to that of *Borrelia burgdorferi* (the agent of Lyme disease), with humans being involved only as incidental "dead-end" hosts (14–17). Exposure to tick bites is considered to be the most common route of human infection, although human granulocytic anaplasmosis has been reported after perinatal transmission or contact with infected animal blood (18,19). *A. phagocytophilum* is associated with *Ixodes* ticks that are known vectors, including *I. scapularis*, *I. pacificus*, and *I. spinipalpis* in the United States (15,20,21), *I. ricinus* mostly in southern, central and northern European regions (22–26), *I. trianguliceps* in the United Kingdom (27), and *Ixodes persulcatus* in eastern parts of Europe (28) and Asia (9).

In Portugal little information is available concerning the epidemiology of *A. phagocytophilum*; the agent was documented only once in *I. ricinus* ticks from Madeira Island (Núncio MS, et al, unpub data). However, the true prevalence and public health impact of *A. phagocytophilum*

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is likely underestimated since little research has been conducted on this bacterium in Portugal. In fact, seasonal outbreaks of enzootic abortions and unspecific febrile illness (commonly named pasture fever) in domestic ruminants, which could be attributable to *A. phagocytophilum*, have been known to breeders and veterinarians across the country for years. Thus, to expand knowledge of *A. phagocytophilum* in Portugal, a detailed investigation was initiated. The preliminary results concerning agent distribution are presented here. The purpose of this study was to investigate both the persistence of *A. phagocytophilum* on Madeira Island, where it was initially described, and the presence of the agent in *Ixodes* ticks from mainland Portugal.

Materials and Methods

Tick Sampling

During 2003 and the beginning of 2004, adults and nymphs were collected from one site on Madeira Island (site 1, Paúl da Serra–Porto Moniz) and from five different sites in the Setúbal District, mainland Portugal (site 2, Barris–Palmela; site 3, Baixa de Palmela; site 4, Picheleiros–Azeitão, site 5, Azeitão, site 6, Maçã–Sesimbra) (Figure 1). Most ticks were unfed, actively questing arthropods; they were obtained by flagging vegetation on pastures and wooded areas bordering farms and country houses. In site 3, additional specimens were also collected from domestic cats (*Felis catus domesticus*). The ticks were identified by morphologic characteristics according to standard taxonomic keys (29,30).

Preparation of DNA Extracts from Ticks

Ticks were processed individually as described (25). Briefly, each tick was taken from the 70% ethanol solution used for storage, air dried, and boiled for 20 min in 100 µL of 0.7 mol/L ammonium hydroxide to free DNA. After cooling, the vial with the lysate was left open for 20 min at 90°C to evaporate the ammonia. The tick lysate was used directly for PCR. To monitor for occurrence of false-positive samples, negative controls were included during extraction of the tick DNA (one control sample for each six tick samples, with a minimum of two controls).

PCR Amplification

DNA amplifications were performed in a Biometra T-3 thermoblock thermal cycler (Biometra GmbH, Göttingen, Germany) with two sets of primers: msp465f and msp980r, derived from the highly conserved regions of major surface protein-2 (*msp2*) paralogous genes of *A. phagocytophilum* (31), and ge9f and ge10r, which amplify a fragment of the 16S rRNA gene of *A. phagocytophilum* (3). PCR was performed in a total volume of 50 µL that contained 1 µmol/L of each primer, 2.5 U of Taq DNA polymerase (Roche, Mannheim, Germany), 200 µmol/L of each deoxynucleotide triphosphate (GeneAmp PCR Reagent Kit, Perkin-Elmer, Foster City, CA), 10 mmol/L Tris HCL, 1.5 mmol/L $MgCl_2$, and 50 mmol/L KCl pH 8.3 (Roche), as described (3,31). Adult ticks were tested individually by using 5 µL of DNA extract. Nymphs were pooled according to geographic site, up to a maximum of 10 different tick extracts per reaction, and 10 µL of the pooled DNA was used for initial screening. All positive pools were confirmed in a second PCR round that used 5 µL of original DNA extract from each nymph. PCR products were separated on 1.5% agarose by electrophorectic migration, stained with ethidium bromide, and visualized under UV light. Quality controls included both positive and negative controls that were PCR amplified in parallel with all specimens. To minimize contamination, DNA preparation with setup, PCR, and sample analysis were performed in three separate rooms.

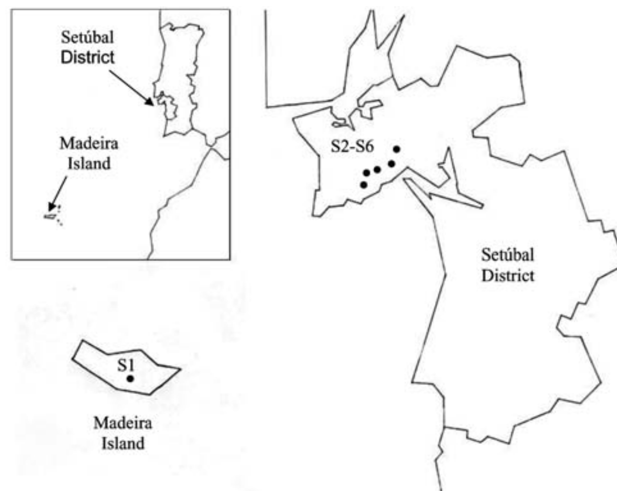

Figure 1. Collection sites in Madeira Island and Setúbal District, mainland Portugal. S, collection site.

DNA Sequencing and Data Analysis

Each positive PCR product was sequenced after DNA purification by a MiniElute PCR Purification Kit (Qiagen, Valencia, CA). For DNA sequencing, the BigDye terminator cycle sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), was used as recommended by the manufacturer. Sample amplifications were performed with the forward and reverse primers used for PCR identification (3,31), with the following modifications: 25 cycles of 96°C for 10 s, 4°C below the melting temperature of each primer for 5 s, and 60°C for 4 min. Dye Ex 96 Kit (Qiagen) was used to remove the dye terminators. Sequences were determined with a 3100 Genetic Analyzer sequencer (Applied Biosystems). After review and editing, sequence homology searches were made by BLASTN

analysis of GenBank. Sequences were aligned by using ClustalX (32) with the neighbor-joining protocol and 1,000 bootstrap replications, and comparing with the 2 *msp2* paralogs of *A. phagocytophilum* Webster strain (AY253530 and AF443404), one *msp2* paralog of USG3 strain (AF029323), and with *A. marginale msp2* (AY138955) and *msp3* (AY127893) as outgroups. Dendrograms illustrating the similarity of *msp2*s were visualized with TreeView (33).

Results

A total of 278 *Ixodes* ticks were tested for *A. phagocytophilum* DNA, including 142 *I. ricinus* from Madeira Island and 43 *I. ricinus* and 93 *I. ventalloi* from Setúbal District. The site of collection, origin, and tick stage are shown in Table 1 and Figure 1. PCR performed with the *msp2* primers detected *A. phagocytophilum* DNA in seven pools of nymphs (six pools of 10 *I. ricinus* from site 1, Madeira Island, and one pool of 4 *I. ventalloi* from site 3, Setúbal District) and also in 1 male *I. ventalloi* from site 3, Setúbal District, as demonstrated by the characteristic 550-bp band. PCRs conducted on individual ticks that comprised positive pools confirmed the results and showed that only one nymph per positive pool contained *A. phagocytophilum* DNA (Tables 1 and 2). PCR test results were negative for all *I. ricinus* collected in the sites in Setúbal District. Overall, the infection rate was 6 (4%) of 142 for *I. ricinus* and 2 (2%) of 93 for *I. ventalloi*. Analysis based on direct amplicon sequencing showed the expected conserved 5′ end followed by ambiguous sequences that corresponded to the hypervariable central region of *msp2*, as anticipated based on the presence of >52 *msp2* copies in the *A. phagocytophilum* HZ strain genome (34). Thus, for appropriate comparison and alignment, the *msp2* 5′ sequences were edited from the positions where unambiguous reads could be determined and terminated 70 nt into the sequence at the approximate beginning of the hypervariable region. A similar alignment protocol for the 3′ end of the *msp2* amplicons showed more ambiguous positions, which prohibited effective alignment and sequence determination. Thus, *msp2* sequence alignments depended upon approximately 70 nt 5′ to the hypervariable region and were performed less for phylogenetic stratification of *A. phagocytophilum* in the ticks than to confirm that the amplified *msp2* sequences were not derived from other related *Anaplasma* or *Ehrlichia* spp. The nucleotide sequences determined for this 70-bp region amplified from all eight ticks showed 98.5%–85.7% similarity, 94.2%–86.9% similarity when compared to representative *msp2* sequences of *A. phagocytophilum* Webster and USG3 strains, and 63.7%–35.0% similarity when compared to *A. marginale msp2* and *msp3* sequences (Figure 2). Sequences obtained from the two *I. ventalloi* from mainland Portugal clustered together and separately from other *msp2* sequences obtained from *I. ricinus* on Madeira Island (Figure 2).

When amplified by using *rrs* primers ge9f and ge10r, compared to *A. phagocytophilum* U02521, sequences were 99% identical to two *I. ventalloi* (636/640 positions and 846/848 positions, respectively) on mainland Portugal and to three *I. ricinus* (836/841, 817/820, and 838/839 positions, respectively) on Madeira Island.

Discussion

This study constitutes part of a larger effort to investigate the distribution of *A. phagocytophilum* in various regions of Portugal. Our data provide supporting evidence that *A. phagocytophilum* is present in actively questing *I. ricinus* from Madeira Island and in *I. ventalloi* from Setúbal District, mainland Portugal.

We used two approaches for identifying *A. phagocytophilum* in ticks: 1) standard amplification of *rrs* that can have limited sensitivity because of a single copy in each bacterial genome, and 2) amplification of *msp2*, a gene for which as many as 52 paralogs are present in the *A. phagocytophilum* genome and for which detection sensitivity is

Table 1. Results of PCR to detect *Anaplasma phagocytophilum* DNA in ticks[a]

Area	Site	Origin	*Ixodes ricinus*			*I. ventalloi*			Total[c]
			Nymphs[b]	F[b]	M[b]	Nymphs[b]	F[b]	M[b]	
Madeira Island									
Paúl da Serra–Porto Moniz	1	Vegetation	6/139	0/2	0/1	–	–	–	142
Setúbal District Portugal Mainland									
Barris–Palmela	2	Vegetation	0/1	0/5	0/7	–	–	0/1	14
Baixa de Palmela	3	Vegetation	0/2	0/2	0/2	1/15	0/6	0/7	34
		Felis catus domesticus	–	–	–	–	0/6	1/4	10
Picheleiros–Azeitão	4	Vegetation	–	0/2	0/2	0/12	0/9	0/18	43
Azeitão	5	Vegetation	–	–	0/1	–	–	0/1	2
Maçã–Sesimbra	6	Vegetation	–	0/10	0/9	0/1	0/4	0/9	33
Total[c]			142	21	22	28	25	40	278

[a]PCR, polymerase chain reaction; F, female; M, male.
[b]Number of positives ticks/number of ticks examined.
[c]Total number of ticks examined.

Table 2. PCR-positive results of ticks[a]

Sites	No. positive nymphs	No. positive adults	Tick extracts codes
Madeira Island			
1	6	–	11; 60; 93; 118; 122; 137
Setúbal District Mainland Portugal			
3	1	1	160; 246 (respectively)

[a]PCR, polymerase chain reaction.

enhanced (34). The pitfall of *msp2* amplification derives from targeting conserved sequences that flank a hypervariable central region, which results in amplicons with partial sequence ambiguity when cloning is not attempted before sequencing (31). These findings are highly unlikely to represent amplicon contamination since marked sequence diversity was observed, and since only a single tick from each pool was positive in each reaction. Although only limited data can gleaned by this analysis, which interrogates only nucleic acids of small size, Casey et al. have shown that *msp2* "similarity" groups, reflecting clusters determined by a similar sequencing approach, can be useful in predicting phylogenetic relationships, particularly with reference to adaptation to specific host niches (35).

Madeira, the main island of the Madeira Archipelago, is located in the North Atlantic Ocean, about 800 km west of the African continent and 1,000 km from the European coast. On this island, *I. ricinus* is the most abundant tick species and the only *Ixodes* tick that was found in this study. *A. phagocytophilum* was detected in 4% of *I. ricinus* collected in Paúl da Serra. Our results corroborate previous findings, although prevalence here is slightly lower than the 7.5% infection rate in ticks previously collected in similar areas (Núncio MS, et al., unpub data). These differences may be attributable to seasonal variations in *A. phagocytophilum* prevalence within reservoir hosts or ticks or to technical aspects of detection. Regardless, studies that use a greater number of samples and that are performed in different seasons, locations, and habitats will be needed to confirm the levels of infection. Nevertheless, these findings are generally similar to those described elsewhere in Europe, although prevalence rates can vary greatly with the origin of *I. ricinus* examined, ranging from a minimum of ≤1% in the United Kingdom, France, and Sweden (23,24,36) to a maximum of 24% to 29% in northern Italy, Germany, and Spain (22,25,26). The public health importance of these findings still remains to be determined. *I. ricinus* is an exophilic, three-host tick known to bite several domestic animals and humans in Portugal (30). Therefore, we can assume that the presence of *A. phagocytophilum* on Madeira Island *I. ricinus* suggests a potential health threat to animals and humans and should be investigated.

Mainland Portugal is the most western region of Europe, with an area of 89,000 km^2, divided into 18 districts. Although *I. ricinus* is not the main tick species in mainland Portugal, it can be found across the country in habitats with favorable conditions. Focused in Setúbal District, to the south of the Tejo River, our study detected *I. ricinus* in all five sites chosen for field work: Barris; Baixa de Palmela; Picheleiros; Azeitão, and Maçã. In those sites, the distribution of *I. ricinus* was accompanied by another *Ixodes* species, *I. ventalloi*. Another ecologically interesting finding that should be further confirmed was that, although all of the *I. ricinus* from mainland Portugal tested negative, evidence of *A. phagocytophilum* was found in 2% of all *I. ventalloi,* including 5% collected in Baixa de Palmela. The *msp2* sequences identified in these two ticks were more closely related to each other than to any *msp2* sequence identified in ticks from Madeira Island. In contrast, *A. phagocytophilum msp2* diversity in *I. ricinus* from Madeira Island was broad and showed overlap with gene sequences identified in North American strains,

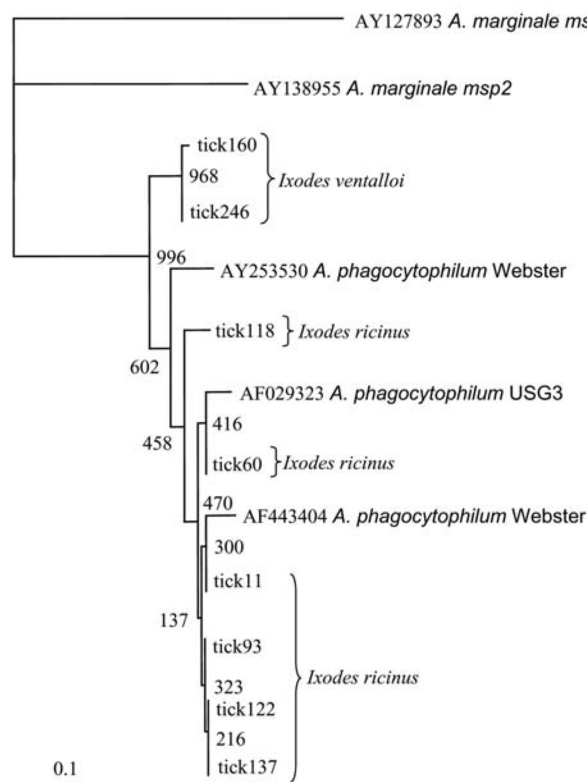

Figure 2. Dendrogram showing the phylogenetic relationships of the msp2 sequences of the newly identified strains and other representative sequences from North American *Anaplasma phagocytophilum* strains (Webster strain–Wisconsin and USG3 strain–eastern United States), and from *A. marginale* Florida strain (*msp2* and *msp3*). Bootstrap values (out of 1,000 iterations) are shown at the nodes. Bar, substitutions/1,000 bp.

as observed for some *A. phagocytophilum* strains in the United Kingdom (35).

To our knowledge, this identification of *A. phagocytophilum* in ticks is the first from mainland Portugal and the first documentation of *Anaplasma* infection in *I. ventalloi*. This species is an endophilic, three-host tick well adapted to a broad range of habitats that vary from open, dry forest in semidesert Mediterranean areas to the mild humid conditions in the southern part of the British Isles. In Portugal, *I. ventalloi* infest a variety of small rodents, carnivores, and lizards but have not been found to feed on humans (30). *A. phagocytophilum* has already been reported in other ticks, besides the known vector species (37–41). The presence in alternate ticks is attributable to the existence of secondary maintenance cycles, in which *A. phagocytophilum* circulates between relatively host-specific, usually nonhuman-biting ticks and their hosts (38,39). Those additional cycles would buffer the agent from local extinction and help reestablish the primary cycles (38,39). Although this hypothesis might explain our results, the competency of *I. ventalloi* to act as vector for *A. phagocytophilum* has yet to be demonstrated. Moreover, the different average prevalences observed in each location suggest that *A. phagocytophilum* is not widely spread in ticks and that some reservoir animals or hosts are needed for its maintenance. Trapping and animal surveillance are needed to provide more information that could help to explain the biological importance of those findings.

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Year-round West Nile Virus Activity, Gulf Coast Region, Texas and Louisiana

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West Nile virus (WNV) was detected in 11 dead birds and two mosquito pools collected in east Texas and southern Louisiana during surveillance studies in the winter of 2003 to 2004. These findings suggest that WNV is active throughout the year in this region of the United States.

Since the initial recognition of West Nile virus (WNV) in North America in 1999 (1), one question that has perplexed epidemiologists and public health officials is how the virus persists during the winter in temperate regions. Arbovirologists and vector biologists have long pondered how arboviruses are maintained during periods when their vectors are absent or inactive (2–5).

For WNV, little information is available on how the virus is maintained in North America during cold periods, when little or no adult mosquito activity occurs. In the winter after the initial 1999 West Nile outbreak in the northeastern United States, Nasci et al. (6) reported detecting West Nile viral RNA and infectious virus in hibernating adult *Culex* mosquitoes collected from underground sanitation tunnels, vacant buildings, and other protective structures in New York City. During the same month (February 2000), another group (7) reported isolating WNV from tissues of a freshly dead hawk found in Westchester County just north of the city. These two observations suggested that, in northern latitudes, WNV may be maintained locally in hibernating *Culex* mosquitoes, as demonstrated earlier for St. Louis encephalitis virus (8), but that low-level virus transmission may also occur during winter.

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The Study

WNV was initially detected in the Houston metropolitan area (Harris County) in the summer of 2002; 105 confirmed human infections with WNV were reported in Houston during the first year (9). In addition, 307 WNV isolates were obtained from dead birds, and 851 WNV-positive pools of *Cx. pipiens quinquefasciatus* were collected during our surveillance studies in the summer and fall of 2002 (9). In 2003, we began a long-term study on the ecology of WNV in Harris County. As part of this program, mosquito and dead bird collections were made by Harris County mosquito control personnel throughout the year. Mosquito collections were made at selected sites throughout the county, with CDC-type light traps and gravid traps (9). Our trapping methods were designed to sample mainly *Cx. p. quinquefasciatus*; this species represented >95% of the mosquitoes collected. After collection, mosquitoes were sorted into pools of <50 females (mean pool size 28.8) and assayed for WNV by an antigen-capture enzyme immunoassay (EIA) (9). Selected EIA-positive mosquito pools (including all winter positives) were confirmed by reverse transcription–polymerase chain reaction (RT-PCR), as described previously (10).

After the initial detection of WNV in Houston in 2002, the Harris County mosquito control personnel established a dead bird surveillance system (9); media reports and public messages instructed county residents to report dead birds, most of which were picked up by the county mosquito control personnel. After collection and species identification, the bird carcasses were frozen at –75°C for subsequent transport to the University of Texas Medical Branch in Galveston, where a sample of brain from each dead bird was cultured for WNV. Culture methods and tests used for virus confirmation were described earlier (9,10). During 2003 and January 2004, the University of Texas Medical Branch group also received a few dead birds from mosquito control districts in Galveston County, Texas, and Louisiana. These avian samples were processed as described above, but are listed separately from the Harris County collections.

Conclusions

Table 1 summarizes the monthly WNV surveillance results for dead birds and mosquitoes tested from Harris County from January 2003 to March 2004. During this 15-month period, 1,623 dead birds, representing 83 avian species, were examined. The number and species of dead birds examined each month varied, reflecting seasonal changes in local avian abundance and deaths, interest of the local citizens in reporting dead birds, winter bird migration into the region, and limits in our ability to process samples. For example, in June, July, and August 2003, a total of 3,352 dead birds were reported by local residents to the

Table 1. Monthly summary of dead birds and mosquitoes tested for West Nile virus (WNV) in Harris County, Texas

Year/month	Mean temperature (°C)[a]	Total birds tested	No. WNV-positive (% positive)	Total mosquitoes tested	No. WNV-positive mosquito pools[b]	WNV minimum infection rate
2003						
January	10.6	46	0	2,164	0	0.00
February	12.5	62	0	1,146	0	0.00
March	16.7	80	0	5,304	0	0.00
April	21.4	98	1 (1.0)	39,000	0	0.00
May	27.1	213	4 (1.9)	58,698	2	0.03
June	27.9	219	33 (15.1)	42,041	40	0.95
July	28.2	205	72 (35.1)	54,582	203	3.72
August	28.9	164	83 (50.6)	40,184	128	3.19
September	25.5	127	38 (29.9)	34,691	21	0.61
October	22.2	73	11 (15.1)	41,465	4	0.10
November	18.7	20	2 (10)	7,562	2	0.13
December	12.6	7	1 (14.3)	8,411	0	0.00
2004						
January	13.2	26	1 (3.8)	12,816	0	0.00
February	12.3	146	3 (2.1)	9,790	0	0.00
March	19.5	137	1 (0.7)	14,714	0	0.00
Total	–	1,623	250 (15.4)	372,568	400	1.07

[a]Mean monthly temperature in Houston (Harris County) (11).
[b]Mean pool size was 28.2 mosquitoes/pool.

county mosquito control staff. During this period, we limited the number of birds tested for WNV to approximately 50 per week, and an attempt was made to sample birds from a variety of different sites within the county. Thus in these 3 months, only 588 dead birds (approximately 17% of the total reported for the period) were actually tested. Also during this period, corvids (Blue Jays and crows) were preferentially selected to be tested, since our experience indicated that these species were most likely to yield virus (9). In contrast, during the winter months (November, December, January, February, and March) fewer dead birds were reported; most of these birds were tested, regardless of species or collection locality. For this reason, Blue Jays (the most common species sampled) represented 35.9% of the birds tested during June, July, and August 2003 but only 7.7% of the birds tested during the months of November, December, January, February, and March.

A similar seasonal bias occurred in our mosquito sampling. During the hottest months of the year (June, July, and August) in Harris County, large numbers of mosquitoes were collected, exceeding our capacity to test them. Thus only a subsample of the mosquitoes collected during this period were tested for WNV. In contrast, during the winter months, adult *Cx. p. quinquesfasciatus* abundance and activity were markedly reduced. During this period, most of the mosquitoes that were collected in traps were assayed for WNV. Our results provide information about the seasonal pattern of WNV activity in the western Gulf region, despite the sampling bias.

Table 2 shows the species composition and WNV infection rates of dead birds collected in Harris County from January 2003 to March 2004. Overall, Blue Jays were the most common dead birds submitted for testing and represented 23.2% of the total; 48.9% of the dead Blue Jays yielded virus. Only 23 American Crows were submitted for WNV testing, but 16 (69.6%) of them were virus-positive upon culture. Crows are much less abundant than Blue Jays in urban areas of the county. Mourning Doves were another commonly submitted dead bird (17.1% of total), but only 2.2% of this species yielded WNV after culture.

As indicated in Table 1, most of the WNV-positive dead birds and mosquitoes from the county were collected during the summer months of June, July, and August. These months are also the three warmest in Harris County (11). However, WNV was also detected in birds and mosquitoes during most other months. Table 3 summarizes the WNV-positive samples identified in our laboratories from November 2003 to March 2004. Ten WNV-positive bird or mosquito samples were from Harris County; the other three positive dead bird samples were submitted from Galveston County, Texas, and Iberia Parish, Louisiana. These isolations of WNV from dead birds and the identification of viral RNA in physiologically active adult mosquitoes collected during the winter season (November–March) imply that the virus is active year-round in Harris County (Table 1) and the western Gulf region. The wide geographic distribution of localities yielding infected birds and mosquitoes also suggests that virus was not restricted to a single community or site but was widespread. Our data from Harris County indicate that peak virus activity occurred mainly during the warm months of the year (June–September), as observed else-

Table 2. West Nile virus–species infection rates among 248 culture-positive dead birds collected in Harris County, Texas, January 2003–March 2004

Common name	Scientific name	Total tested	No. infected (%)
Blue Jay	*Cyanocitta cristata*	376	184 (48.9)
American Crow	*Corvus brachyrhynchos*	23	16 (69.6)
Loggerhead Shrike	*Lanius ludovicianus*	14	7 (50)
House Sparrow	*Passer domesticus*	119	19 (16.0)
Northern Mockingbird	*Mimus polyglottus*	99	8 (8.1)
Mourning Dove	*Zenaida macroura*	278	6 (2.2)
Rock Dove	*Columba livia*	48	1 (2.1)
Inca Dove	*Columbina inca*	38	1 (2.6)
Great-crested Flycatcher	*Myiarchus crinitus*	1	1 (100)
Carolina Chickadee	*Parus carolinensis*	3	1 (33.3)
Tufted Titmouse	*Baeolophus bicolor*	2	1 (50)
Common Grackle	*Quiscalus quiscula*	84	3 (3.6)
Orchard Oriole	*Icterus spurius*	3	1 (33.3)
American Goldfinch	*Carduelis tristis*	1	1 (100)

where in North America (12), but that low-level virus activity continued during the rest of the year in this region. Prolonged periods with temperatures <0°C are uncommon in Harris County and the western Gulf Coast, so *Cx. p. quinquefasciatus* (the presumed vector of WNV in the region) does not enter a true diapause, as does its northern counterpart, *Cx. pipiens* (13). Our field observations in Harris County indicate that *Cx. p. quinquefaciatus* adults become relatively inactive during cold periods, resting under buildings and in storm drains and sewers; however, these mosquitoes become active again during warm periods in the winter months. The fact that adults can be captured in light traps and eggs laid in gravid traps throughout the year in the county (Table 1) is evidence of their continual activity. This intermittent host-seeking activity throughout the winter probably accounts for continued low-level WNV transmission and infection in the resident avian population. On the basis of these observations, we believe that this mechanism is probably the principal one by which WNV overwinters and persists in the western Gulf region of the United States.

The results of our study also confirm observations by others (14) that surveillance of dead birds is a sensitive method for detecting early WNV activity. The use of sentinel animals (in this case dead birds) is a well-established method of arbovirus surveillance and sometimes detects virus activity during periods when none can be detected in mosquitoes (15). The presumed increased sensitivity of dead bird surveillance may explain why a few bird isolates of WNV were obtained each month during winter, but no virus activity was detected in *Cx. p. quinquefaciatus* during the same period.

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Table 3. Confirmed West Nile virus (WNV) activity in birds and mosquitoes collected in east Texas and southern Louisiana

Date collected	WNV-positive sample	Locality
Nov. 3, 2003	Blue Jay	Santa Fe, Galveston Co., TX
Nov. 7, 2003	Blue Jay	Dickinson, Galveston Co., TX
Nov. 14, 2003	Blue Jay	Houston, Harris Co., TX
Nov. 18, 2003	Blue Jay	Pasadena, Harris Co., TX
Nov. 20, 2003	*Culex p. quinquefasciatus* pool	Houston, Harris Co., TX
Nov. 20, 2003	*Cx. p. quinquefasciatus* pool	Houston, Harris Co., TX
Dec. 16, 2003	American Crow	Spring, Harris Co., TX
Jan. 2, 2004	Northern Cardinal	New Iberia, Iberia Parish, LA
Jan. 22, 2004	Blue Jay	Tomball, Harris Co., TX
Feb. 12, 2004	American Goldfinch	Houston, Harris Co., TX
Feb. 12, 2004	American Crow	Kingwood, Harris Co., TX
Feb. 19, 2004	Loggerhead Shrike	Houston, Harris Co., TX
Mar. 4, 2004	American Crow	Kingwood, Harris Co., TX

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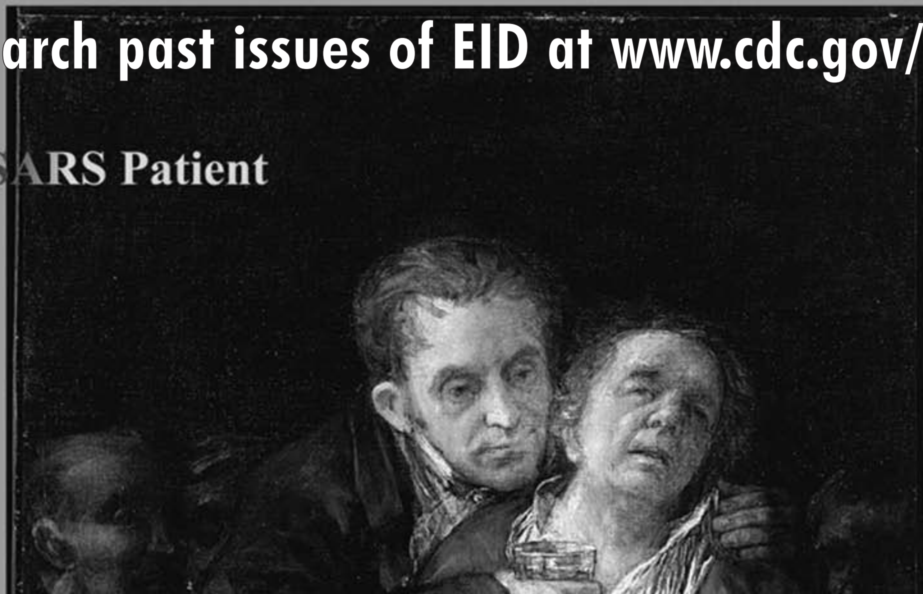

SARS-CoV Antibody Prevalence in All Hong Kong Patient Contacts

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A total of 1,068 asymptomatic close contacts of patients with severe acute respiratory (SARS) from the 2003 epidemic in Hong Kong were serologically tested, and 2 (0.19%) were positive for SARS coronavirus immunoglobulin G antibody. SARS rarely manifests as a subclinical infection, and at present, wild animal species are the only important natural reservoirs of the virus.

Since severe acute respiratory syndrome (SARS) and the coronavirus (SARS-CoV) that causes it have emerged and spread, considerable progress has been made in understanding the biology, pathogenesis, and epidemiologic features of both the virus and the disease. Epidemiologic studies of hospitalized patients suggest that the overall transmissibility of SARS (as indicated by the basic reproductive number $R_0 = 2.7$, 95% confidence interval [CI] 2.2–3.7) (1) is relatively low compared to other pathogens. However, such studies could not take into account possible episodes of mild or moderate illness that did not require inpatient medical care and could not address whether asymptomatic community spread played a role in the 2003 epidemic. If this type of spread occurred, sufficient herd immunity against SARS-CoV to protect against another large-scale outbreak might have been developed in the population. The full spectrum of disease associated with SARS-CoV infection should be examined to define more precisely what constitutes a case requiring quarantine and isolation to minimize potential human-to-human spread. Understanding these issues requires the systematic study of the seroprevalence of SARS-CoV antibody in a large sample stratified by age and other baseline characteristics, especially since children were disproportionately less affected by SARS, both in terms of reduced incidence and severity of infection. Serologic surveys can be based on a random sample from the total population with appropriate stratification, on serum collected for other reasons (e.g., blood donors, all hospital admissions), or on surveys of persons who resided in sites of superspreading events or who have had close contact with a confirmed SARS patient.

We report a serologic survey for immunoglobulin (Ig) G against SARS-CoV in a representative sample of close contacts of all SARS patients in Hong Kong (>76% had laboratory confirmation of SARS by either paired serology or repeat reverse transcription–polymerase chain reaction (RT-PCR) according to World Health Organization [WHO] criteria [2]).

The Study

During the epidemic, close contacts were prospectively identified by the Hong Kong Special Administrative Region Government Department of Health through standardized telephone interviews with all 1,755 confirmed SARS patients within 1 week of hospital admission (February 15–June 22, 2003). A close contact was defined as a person who had cared for, lived with (in the same household), or came into direct contact with body fluids of the SARS patients within 10 days before hospital admission. A total of 3,612 close contacts were recorded; 505 were diagnosed as having SARS. Of the remaining 3,107 contacts, 2,805 (90%) had a telephone number available, as reported by the primary patient. We successfully contacted 2,337 (83%) from October 23 to November 30, 2003, and 1,776 (57% of those eligible) consented to a telephone interview after the purpose of the study was explained to them by trained public health nurses. The interview consisted of questions that assessed the relationship between the patients and contacts; the timing, intensity and frequency of contact; precautionary measures adopted during contact with the patient; known contact with other SARS patients; clinical symptoms of febrile, respiratory, gastrointestinal, or constitutional illness since February 2003; medical and travel history; and sociodemographic details. Participants were then invited to provide blood samples for serologic testing. Shopping coupons (worth U.S. $25.00) were given to participants after blood was collected as compensation for time and travel costs.

Samples were screened by the Government Virus Unit of the Department of Health by using viral lysate enzyme-linked immunosorbent assay (ELISA) (GBI Biotech, Beijing). Positive results were confirmed with immunofluorescence assay (IFA) and neutralization tests. For the IFA, microscopic slides coated with SARS-CoV–infected

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FRhK4 cells were incubated with serum samples at serial twofold dilutions starting from1:25. A positive test is indicated by cytoplasmic fluorescence under UV microscopy. By using IFA as the standard, the ELISA detects antibody with IFA titer of ≥25 (i.e., sensitivity of 100%) and has a specificity of 95%. Neutralization test was performed by standard virologic method with Vero E6 cells and SARS-CoV isolate 6109. A titer of ≥10 was considered positive. The reported sensitivity of 100% was for convalescent-phase serum samples taken a few weeks after the onset of infection in SARS patients, which should apply to our study. During the early phase of infection, IgM predominates; the ELISA kit we used detects IgG only. Therefore, the sensitivity is 80%–90% (depending on the number of days after illness onset when the serum samples were taken). However, this sensitivity should not have affected our findings, which were based on tests carried out at least 6 months after the last reported case of SARS in Hong Kong. The study received ethics approval from the Department of Health Ethics Committee, which complies with the Declaration of Helsinki.

Results and Conclusions

Of the 1,068 samples analyzed, 2 (0.19%, exact 95% CI 0.02%–0.67%) had a positive titer (1:25 to 1:50 on IFA compared to at least 1:100 in most recovered SARS cases) for SARS-CoV IgG antibody. Neither participant with a positive sample reported a chronic medical condition or being sick with febrile or respiratory illness from February to August. Both seropositive participants arose from two superspreading events in Hong Kong, i.e., Prince of Wales Hospital nosocomial outbreak and Amoy Gardens environmental point source community outbreak (1,3). The contact of the Prince of Wales Hospital seropositive participant reported one other close contact, who was interviewed but declined to be tested. The other seropositive index patient living in Amoy Gardens was separately identified by three intrafamilial index patients, all of whom lived in the same household and reported only each other as close contacts. The participants who consented to testing were broadly similar to those who declined, except that the first group had relatively fewer children and fewer of the first group were men (Table). However, those who declined testing were more likely to report more frequent contact and closer relationships with SARS patients, more febrile or respiratory illness episodes since February, and a travel history to SARS-affected regions, which may have biased our seroprevalence estimate upwards.

The extent of seroconversion in close contacts of confirmed patients should provide the upper limit of SARS-CoV antibody seroprevalence in the general population, given the relatively intense exposure history of these persons to SARS patients. Our finding of the near absence of transmission resulting in asymptomatic infection in this representative high-risk group of close contacts indicates that the prevailing SARS-CoV strains in Hong Kong almost always led to clinically apparent disease. Whereas some SARS patients, especially healthcare workers, might have been initially admitted to reduce transmission to family members, virtually all SARS patients (perhaps with very few exceptions in children [4]) had severe disease requiring inpatient treatment; thus, we can infer that infection with SARS-CoV inevitably caused severe disease requiring hospitalization.

Although our results suggest that SARS-CoV was a new virus in humans without a close precursor or an antigenically related virus that would have induced at least a small degree of cross-reactivity on serologic testing, a recent study on a select group of 938 healthy Hong Kong adults, whose serum had been previously stored as part of a hepatitis B serosurvey in 2001, indicated that 1.8% of the sample had acquired a SARS-CoV–related virus infection at least 2 years before the 2003 SARS outbreak (5). The investigators speculated that the virus that affected these healthy, seropositive persons was antigenically closer to the recently isolated animal SARS-CoV–like virus (3) than human SARS-CoV, but interspecies transmission from animals to humans was probably inefficient, as the virus might not have adapted in the new host. This hypothesis would explain why only a few persons became infected and why they were likely to have been asymptomatic. This hypothesis would be compatible with the presumed asymptomatic infection observed in Guangdong animal traders, especially in those who handled masked palm civets, who had a seropositivity rate of 72.7% (exact 95% CI 49.8%–89.3%) in the absence of prior overt clinical disease (6).

The limitations of the study include incomplete contact tracing, especially in the earlier parts of the epidemic, and potential recall bias from underreporting of contacts by some patients who were too sick to answer questions. Another possible shortcoming is the lack of a survey of close contacts who did not report a telephone number, although there is no reason to suspect they had a systematically different serologic profile. In fact, these were mostly nonhousehold contacts who would have had less intense exposure to SARS patients. In addition, because peak infectivity, as indicated by viral load, usually occurred during week 2 of illness (7), when most of the patients would have been isolated in hospital (the mean onset-to-admission interval decreased from a maximum of 9.3 days in late February to 1.0 day by mid-May) (8), transmission to close contacts in the later stages of the epidemic was less likely. Finally, contacts who refused to participate (561) or refused to have serologic testing (708) might have done so because they were concerned about having had SARS

Table. Characteristics of close contacts recalled for serologic testing (N = 1,776)

Characteristic	Tested for IgG against SARS-CoV, n = 1,068 (%)	Declined antibody testing, n = 708 (%)	p value
Age (y)			< 0.001
≤10	53 (5.0)	126 (18.1)	
11–17	77 (7.2)	68 (9.7)	
18–44	515 (48.3)	278 (39.8)	
45–64	330 (30.9)	138 (19.8)	
≥65	92 (8.6)	88 (12.6)	
Sex			0.02
Female	579 (54.2)	341 (48.3)	
Male	489 (45.8)	365 (51.7)	
Travel history to SARS-affected areas since February, 2003[a]			< 0.001
Yes	523 (49.0)	268 (37.9)	
No	545 (51.0)	440 (62.1)	
Relationship with SARS case			0.001
Household family member	789 (74.4)	499 (70.5)	
Non-household family member or relative	230 (21.7)	164 (23.2)	
Friend/classmate/colleague	25 (2.4)	12 (1.7)	
Other (e.g., domestic helper)	16 (1.5)	33 (4.7)	
Frequency of contact with SARS patient within 10 days of hospital admission			0.06
Daily	666 (62.6)	405 (57.9)	
4–6 days per week	82 (7.7)	56 (8.0)	
1–3 days per week	161 (15.1)	103 (14.7)	
Very occasionally	155 (14.6)	135 (19.3)	
No. of precautions adopted during SARS outbreak[b]			0.25
≤2	60 (6.6)	47 (8.6)	
3–4	113 (12.4)	81 (14.8)	
5–6	334 (36.7)	187 (34.1)	
7–8	402 (44.2)	234 (42.6)	
No. of febrile or respiratory illness episodes since February 2003			0.02
0	643 (61.7)	471 (68.4)	
1–2	351 (33.7)	193 (28.0)	
≥3	48 (4.6)	25 (3.6)	
Presence of chronic medical conditions			0.10
Yes	270 (28.3)	149 (24.6)	
No	683 (71.7)	457 (75.4)	
Self-perceived health status in previous week			0.34
Excellent	124 (11.6)	84 (12.0)	
Very good	317 (29.7)	222 (31.8)	
Good	323 (30.3)	223 (31.9)	
Fair	279 (26.1)	152 (21.7)	
Poor	24 (2.2)	18 (2.6)	

[a]Includes Canada, China, Singapore, and Taiwan.
[b]Includes washing hands before touching mouth, eyes, and nose; washing hands with soap; wearing face mask; using serving utensils during meals; adopting precautionary measures when touching possibly contaminated objects, washing hands after touching possibly contaminated objects; adopting home preventive measures (such as maintaining good ventilation and using bleach to clean surfaces and home appliances) against SARS; and adopting workplace preventive measures (such as maintaining good ventilation, using bleach to clean surfaces and office furniture, and not allowing staff who are sick to come to work) against SARS.

(possibly because of having had SARS-like symptoms) and did not want to be identified and stigmatized as having been infected with SARS-CoV. Surveys in other countries with large-scale outbreaks such as Canada, China, Singapore, and Taiwan should be undertaken to confirm our findings.

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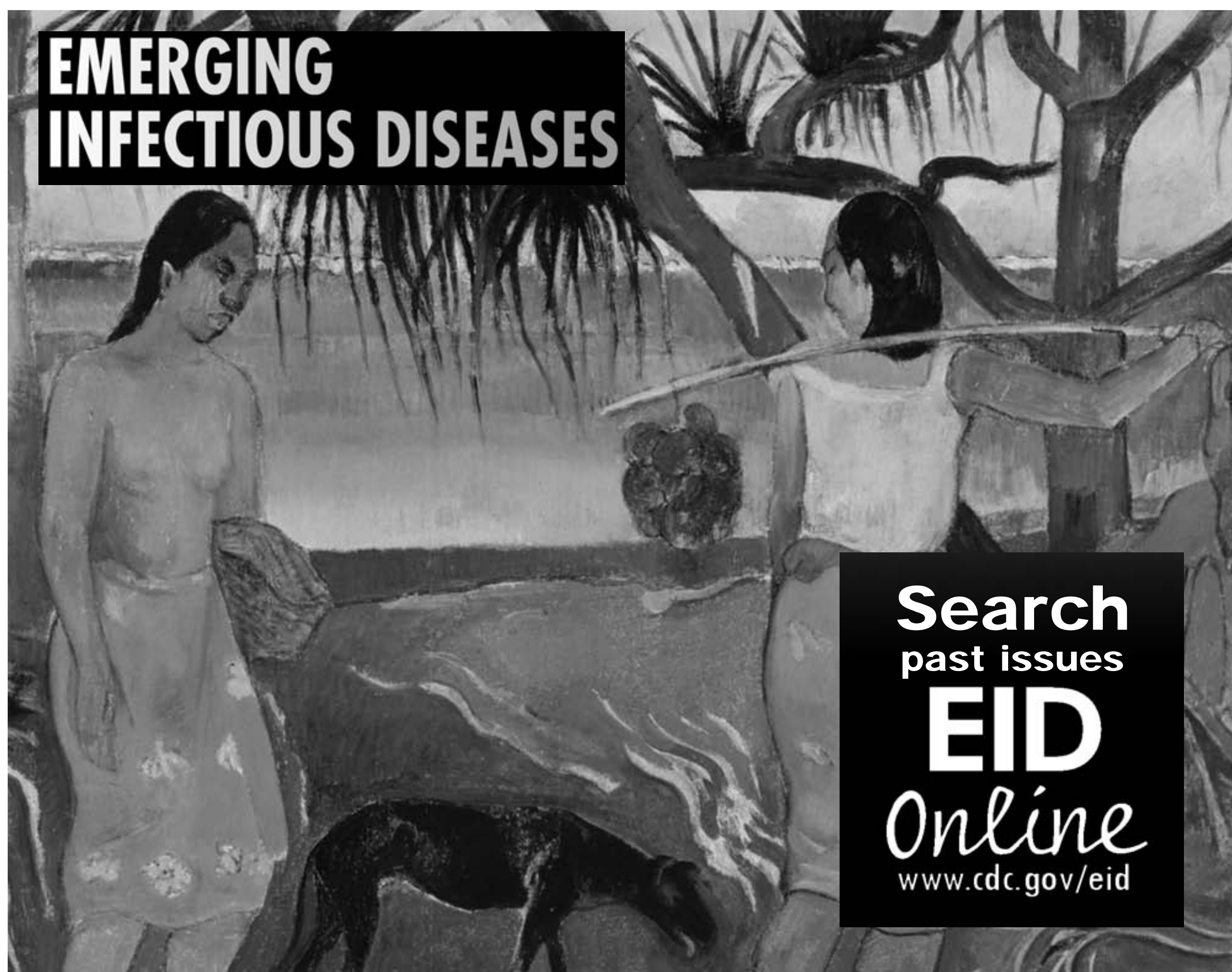

Yellow Fever Virus Infectivity for Bolivian *Aedes aegypti* Mosquitoes

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The absence of urban yellow fever virus (YFV) in Bolivian cities has been attributed to the lack of competent urban mosquito vectors. Experiments with *Aedes aegypti* from Santa Cruz, Bolivia, demonstrated infection (100%), dissemination (20%), and transmission of a Bolivian YFV strain (CENETROP-322).

Yellow fever virus (YFV) may cause severe hemorrhagic fever in humans. The virus is transmitted between susceptible vertebrate hosts by infected mosquitoes in the genera *Aedes*, *Haemagogus*, or *Sabethes* (1). In the Americas, YFV occurs in two transmission cycles. In the jungle/sylvatic cycle, the virus is transmitted between susceptible monkeys, and possibly other vertebrates, by tree-hole–breeding mosquitoes (1). Jungle yellow fever (YF) cases occur when these infected vectors feed on susceptible humans. In the urban cycle, YFV is transmitted to humans by *Aedes aegypti* mosquitoes (1). In 2003, a total of 226 cases of jungle YF were reported from South America to the Pan American Health Organization, and as of June 23, ongoing outbreaks in Bolivia, Brazil, Colombia, and Peru during 2004 have thus far resulted in 86 confirmed cases and 41 deaths (2).

An *Ae. aegypti* eradication campaign initiated by the Pan American Sanitary Bureau in 1947 eliminated this species from most of Central and South America, and urban YF disappeared from the Americas in the 1940s. However, during the past 20 years, many countries abandoned *Ae. aegypti* control measures, and this urban vector now reoccupies almost the entire area of its distribution preeradication (1). *Ae. aegypti* was eradicated from Bolivia during the 1960s and 1970s but reappeared in the city of Santa Cruz in 1980, and epidemics of dengue fever occurred during the 1980s and 1990s (3). In 1997 to 1998, six cases of YF were reported among Santa Cruz residents, and some were regarded as urban YF cases (3); despite a population >1 million, low vaccine immunization coverage, and the presence of *Ae. aegypti* (3), no urban YF outbreak occurred. Based on these observations, researchers have suggested that sylvan strains of YFV circulating in Bolivia may not be infective for Bolivian *Ae. aegypti*. This study examined that hypothesis and the infectivity of a Bolivian strain of YFV for Bolivian *Ae. aegypti.*

The Study

All work involving infectious YFV was performed in biosafety level 3 facilities at the University of Texas Medical Branch. Three human isolates of YFV were used: CENETROP-322 (La Paz Department, Bolivia, 1999), Jimenez (Panama, 1974), and Asibi (Ghana, 1927). To facilitate transmission from a viremic vertebrate, viruses were adapted by serial passage through Syrian golden hamsters (*Mesocricetus auratus*), following the model of Tesh et al. (4,5); CENETROP-322 and Jimenez were passaged 11 times, and the Asibi strain was passaged 10 times.

The SC strain of *Ae. aegypti* was started with mosquitoes collected from Santa Cruz, Bolivia, in 2001. Mosquitoes used in this experiment were from laboratory-reared F2-F3 generation. The REX-D strain, an old laboratory colony originally started with mosquitoes collected in Rexville, Puerto Rico and of previously defined susceptibility to YFV infection (6) was used as a control. Mosquitoes were maintained as previously described (7).

Three hamsters were injected intraperitoneally (IP) with 100 µL of clarified liver homogenate, which contained approximately 10^6 $\log_{10}$ tissue culture infectious dose 50% ($TCID_{50}$/mL) of each YFV strain. Three days after infection, when viremia levels have been shown to peak (4), hamsters were anesthetized (50 mg *Pentobarb*/kg IP) and simultaneously exposed to 10-day-old *Ae. aegypti* SC or REX-D mosquitoes for 1 h. Fully engorged mosquitoes in each group were placed in separate cages and incubated for 15 days at 28°C and 80% relative humidity on a diet of 10% sucrose. Hamster blood samples were collected immediately afterward and stored at –80°C for viral assay.

Virus Transmission

At day 15 after infection, mosquitoes were allowed to feed on 8-day-old mice. (Mice were used in preference to adult hamsters because they are more susceptible to fatal infection.) After feeding, mosquitoes were assayed for YFV infection and dissemination by whole-body titration and immunofluorescence assay (IFA) of head-squash material, respectively (7). For IFA, a broadly reactive anti-flavivirus monoclonal antibody (813) with biotin-streptavidin amplification was used (8). Suckling mice were observed for illness and death. The brains of two paralyzed mice were tested for viral antigen by culturing on Vero cells. At day 14 after exposure, serum specimens from

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surviving mice were tested for anti-YFV antibodies by hemagglutination inhibition (HI) test (9).

All three strains of YFV caused viremia in hamsters on day 3 after infection, with titers of 8.5, 8.7, and 7.3 $TCID_{50}log_{10}$/mL (CENETROP-322, Jimenez, and Asibi, respectively), as determined by assay in mosquito cell cultures (4). Determination of mosquito infection and dissemination rates showed that all YFV strains were able to infect both strains of *Ae. aegypti*, although infection rates varied from 15.1% to 100%. Mean total mosquito YFV titers were relatively low, but the presence of virus after 15 days, evidence of dissemination in the insect, and transmission data are all indicative of replication. At day 15 postinfection, 100% of SC *Ae. aegypti* were infected with CENETROP-322. Infection rates and mean viral titers for CENETROP-322 were higher in SC *Ae. aegypti* than in the REX-D strain (Table 1). Infection rates of CENETROP-322 and Jimenez were higher than the rate for Asibi in both mosquito strains.

Virus titers in the mosquitoes varied considerably but were lowest in REX-D strain insects infected with CENETROP-322 (Table 1). These mosquitoes had the highest dissemination rates (80.7%), which indicates little correlation between virus titer and dissemination rates. Dissemination rates were highest in the REX-D strain; but our data demonstrate that both Panamanian and Bolivian strains of YFV disseminated in Santa Cruz *Ae. aegypti* (Table 1).

Transmission trials used 8-day-old mice to feed mosquitoes that had ingested YFV 15 days earlier (Table 2). Five mice were used per virus strain. The reluctance of mosquitoes to feed on suckling mice precluded an evaluation of all YFV-mosquito combinations. However, HI results indicated that antibodies against YFV (320 titer, Table 2) developed in one mouse exposed to SC mosquitoes infected with CENETROP-322, which indicated transmission by the Bolivian *Ae. aegypti*. In addition, Jimenez and Asibi strains of YFV were transmitted by the REX-D mosquitoes. Transmission was confirmed by recovering YFV by culture from dead mice.

Conclusions

Susceptibility to YFV infection is highly variable in mosquitoes from different locations (10–12) and may be influenced by selection (6) and colonization (13). Although the use of Bolivian mosquitoes with few laboratory-reared generations compromised our ability to use large numbers, obtaining competence data as possibly representative of wild, noncolonized, mosquitoes was important. Dissemination and transmission by Santa Cruz *Ae. aegypti* indicate their ability to serve as vectors for a Bolivian strain of YFV. A critical component of this study was the use of a hamster model for YFV (4). The high viremia levels in hamsters (4,5) facilitate oral infection of mosquitoes and more closely resemble natural infection than feeding the insects on artificial blood meals. Suckling mice remain useful because of their sensitivity to YFV infection.

We could argue that by passing the virus in hamsters, the virus phenotype may be altered with respect to vector infectivity. However, after equivalent passages, the infectivity of the Bolivian, Panamanian, and African strains differed. The Jimenez strain was highly infectious for Bolivian *Ae. aegypti* (93.5%), with a relatively high dissemination rate (34.5%). In contrast, the Asibi was relatively noninfectious for the Bolivian mosquitoes. Considering the numbers of mosquitoes and virus strains involved, we cannot conclude that this finding reflects a general trend of incompatibility between South American *Ae. aegypti* and YFV of African origin. However, the results obtained are in close agreement with the findings by Tabachnick et al. (12). Johnson et al. (14), using Brazilian strains of *Ae. aegypti* and YFV, reported similar results of 35% infection rates and 25% dissemination rates. Lourenço-de-Oliveira et al. (11,15) observed infection rates from 0% to 48.6% in Brazilian *Ae. aegypti* infected with Brazilian YFV. In comparison, we found higher infection rates for Panamian and Bolivian YF viruses (63.3%–100%), but this finding may reflect our use of a viremic animal to infect the mosquitoes, whereas Tabachnick et al. (12) and Johnson et al. (14) used artificially prepared blood meals. Our results also demonstrate

Table 1. Infection, dissemination, and virus titers for three strains of yellow fever virus, CENETROP-322, Jimenez, and Asibi, in two strains of *Aedes aegypti* mosquitoes, Santa Cruz and REX-D, at day 15 postinfection[a]

Ae. aegypti	Virus strain	Hamster serum titer ($TCID_{50}$ log_{10}/mL)	No. infected by titration (5%)	Mean titer of positives ($TCID_{50}$ log_{10}/mL)	Dissemination rate by IFA (%)
Santa Cruz	CENETROP-322	8.5	10/10 (100)	3.5	20
Santa Cruz	Jimenez	8.7	29/31 (93.5)	3.5	34.5
Santa Cruz	Asibi	7.3	3/26 (15.1)	3.5	0
REX-D	CENETROP-322	8.5	19/30 (63.3)	1.5	80.7
REX-D	Jimenez	8.7	27/30 (90)	3.5	73.4
REX-D	Asibi	7.3	13/30 (43.3)	4.0	38.4

[a]$TCID_{50}$, tissue culture infectious dose 50%; IFA, immunofluorescence assay.

Table 2. Transmission of yellow fever virus by infected *Aedes aegypti* mosquitoes to suckling mice[a]

Ae. aegypti strain	Virus strain	No. mice infected/ No. exposed (%)	HI titer for 4 U of antigen		
			YFV	SLEV	WNV
Santa Cruz-	CENETROP-322	1/5 (20)[b]	0	0	0
			0	0	0
			320	0	0
			0	0	0
			0	0	0
REX-D	Jimenez	4/5 (80)[c]	NT	NT	NT
			NT	NT	NT
			NT	NT	NT
			NT	NT	NT
			NT	NT	NT
REX-D	Asibi	3/5 (60)[b]	160	0	0
			0	0	0
			0	0	0
			80	0	0
			40	0	0

[a]HI, hemagluttination-inhibition; YFV, yellow fever virus; SLEV, St. Louis encephalitis virus; WNV, West Nile virus; NT, not tested.
[b]Infection determined by presence of anti-YF antibodies in mice sera (HI test). 0 indicates a titer of <1:20.
[c]Infection determined by death/virus detection for suckling mice.

that passaging YFV in hamsters does not compromise the ability of the virus to infect mosquitoes and that the hamster model is useful to study mosquito competence for YFV.

In conclusion, our results do not support the hypothesis that Bolivian strains of YFV cannot infect Bolivian *Ae. aegypti* and demonstrate that the recolonizing (after 1980) South American strains of *Ae. aegypti* are potential YFV vectors. The reason urban YF epidemics have not yet occurred in South America, including in the city of Santa Cruz, Bolivia, where some cases were recently reported within the city limits (3), is still unknown. The mosquito infection rates observed in our study were higher that those reported by Lourenço-de-Oliviera et al. (11), and we also demonstrated YFV transmission (albeit at a low level). Thus, if YFV were to be reintroduced into urban areas of South America, a transmission cycle could possibly be established. The absence of epidemic YF may be the result of other factors, including widespread deforestation and less opportunity for YFV to move out of the sylvatic cycle, better mosquito control, the local population's YFV vaccine status, and, possibly, heterologous antibodies to other flaviviruses such as dengue (5).

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Typing of *Borrelia* Relapsing Fever Group Strains

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Partial sequencing of the 16S-23S rDNA intergenic spacer showed two to four genotypes each for *Borrelia hermsii* and *B. turicatae*, both relapsing fever agents transmitted by argasid ticks, and for *B. miyamotoi* and *B. lonestari*, transmitted by ixodid ticks. Field surveys of *Ixodes* ticks in Connecticut and Sweden showed limited local diversity for *B. miyamotoi*.

The two major clades of species in the genus *Borrelia* are the Lyme borreliosis group and the relapsing fever group (1). The Lyme borreliosis group includes *Borrelia burgdorferi*, *B. afzelii*, *B. garinii*, and several other species not associated with human disease. The relapsing fever group includes several species, such as *B. hermsii* in the Nearctic ecologic region and *B. persica* in the Palearctic, that cause endemic relapsing fever in humans (2). The known relapsing fever agents are transmitted by soft (argasid) ticks, usually an *Ornithodoros* species. In 1995, *B. miyamotoi* was first isolated from *Ixodes persulcatus* hard (ixodid) ticks in Japan (3). Genomic DNA of the newly identified spirochete cross-hybridized to a greater extent with DNA of relapsing fever species than with DNA of Lyme borreliosis species. In 1996, *B. lonestari* was discovered in *Amblyomma americanum*, an ixodid tick of the southern and eastern United States (4,5). Although *B. lonestari* is associated with a Lyme borreliosis–like disorder in the southern United States (6), sequence analysis showed that *B. lonestari*, like *B. miyamotoi*, was in a clade with the relapsing fever group rather than the Lyme borreliosis group (4,5). More recently, spirochetes closely related to *B. miyamotoi*, and provisionally designated here as *B. miyamotoi* sensu lato (s.l.), were discovered in *I. scapularis* ticks in the United States (7) and *I. ricinus* ticks in Europe (8).

The Study

The public health importance of the newly discovered species remains to be determined. However, finding *B. miyamotoi* s.l. in *I. scapularis*, *I. ricinus*, and *I. persulcatus*, the predominant vectors of Lyme borreliosis in North America, Europe, and Asia, respectively, complicates interpreting epidemiologic studies of Lyme borreliosis and other ixodid-borne disorders. A method to identify and distinguish strains within species is needed to carry out studies of the population biology and of the possible etiologic roles of these organisms. Since most of these microorganisms are to date uncultivable or poorly cultivable, a method using DNA amplification by polymerase chain reaction (PCR) is preferable. On the basis of the findings of Liveris et al. (9), we further developed sequence analysis of the 16S-23S rRNA intergenic spacer (IGS) for strain typing and showed its advantages over other loci for the Lyme borreliosis agents *B. burgdorferi* and *B. afzelii* (10). For this study, we applied this approach to typing the new *Borrelia* spp. and included two relapsing fever agents, *B. hermsii* (endemic in the western and northwestern United States) and *B. turicatae* (endemic in the southwestern and south-central United States) (2).

Nine isolates of *B. hermsii* in our culture collection originated in New Mexico, Colorado, California, and Washington State and were either from *Ornithodoros hermsi* ticks, patients with relapsing fever, or, in one case, a bird (11). Two *B. turicatae* isolates were from *O. turicata* ticks from Texas and Kansas. *B. miyamotoi* strains HT24, HT31, and HK004 from *I. persulcatus* ticks and strains NB103-1 and FR64b from *Apodemus* spp. mice were from Hokkaido, Japan (3,12). Cultivable strains of these species were grown in Barbour-Stoenner-Kelly II medium. Uncultivated species were initially identified in total DNA extracts of ticks by using *Borrelia* genus–specific PCR, targeting *flaB* gene (5). Approximately 2% of *A. americanum* nymphs and adult females in collections from different areas of New Jersey, Illinois, and Missouri contained *B. lonestari*. *B. miyamotoi* s.l. spirochetes were identified in *I. scapularis* nymphs collected at a 7.2-ha field site in southern Connecticut and in *I. ricinus* nymphs collected at a 1.5-ha site in Blekinge County in Sweden (10). A Connecticut strain of *B. miyamotoi* s.l. strain was maintained in *Mus musculus* (7).

Part of the intergenic spacer was amplified by PCR with primers for the 3′ end of the 16S rRNA gene and the *ileT* tRNA gene (10). As a comparison to the intergenic spacer locus and to assess linkage disequilibrium, we also partially sequenced the chromosomal gene for the P66 outer membrane protein (10,13) after amplification by PCR as described in the Table footnotes. The PCR products were either directly sequenced or first cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) before sequencing on a Beckman CEQ 8000 (Beckman Coulter,

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Fullerton, CA) automated sequencer. The sequences were aligned automatically by using Clustal X software (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX) and then manually with MacCLADE version 4.05 (http://macclade.org/macclade.html) (10). The maximum lengths of the alignments (http://spiro.mmg.uci.edu/data) were set by the shortest available sequence. Accession numbers for the deposited sequences are given in the legend for the Figure and in a footnote for the Table.

The PCR products for the intergenic spacer locus varied in length between species and ranged from 388 bp for *B. miyamotoi* s.l. from Sweden to 685 bp for *B. turicatae*. The PCR product for the *p66* gene was 605–614 bp between species. The Table summarizes the statistics for the aligned intergenic spacer and *p66* sequences of the *B. miyamotoi* s.l., *B. lonestari*, and *B. hermsii*. The mean nucleotide diversity normalized for each aligned position was 38%–130% higher for the intergenic spacer locus than for the *p66* locus. At the same time, intragenic recombination was not detected at the intergenic spacer locus with Sawyer's test (www.math.wustl.edu/~sawyer/mbprogs), which assesses the likelihood that polymorphisms in a sequence arose through recombination rather than mutation (data not shown). This result was consistent with the undetectable recombination at the intergenic spacer loci of *B. burgdorferi* (10).

The genetic diversity at the intergenic spacer and *p66* loci for the relapsing fever group species in a given geographic area was more limited than was the case for Lyme borreliosis species (10). This limitation was most apparent with the *B. miyamotoi* s.l. sequences of 22 samples from Connecticut and 6 samples from Sweden. As shown by the phylogram (Figure), only one intergenic spacer genotype each was found for *B. miyamotoi* s.l. from the Connecticut site and from Sweden. In contrast, collections at the same sites and times, and from the same tick vectors, provided 8 intergenic spacer genotypes among 62 *B. burgdorferi* samples in *I. scapularis* and 9 intergenic spacer genotypes among 73 *B. afzelii* samples in *I. ricinus* (10). Accepting a type I error level of 0.05, we would expect to have detected a second genotype of *B. miyamotoi* s.l. in a sample size of 22 if its proportion was ≥14%. The findings at the *p66* locus for 10 Connecticut samples and for 4 samples from Sweden were similar: only one *p66* genotype was detected at each location.

The samples of the other relapsing fever group species were not prospectively acquired for population studies, and thus, the findings provide only a tentative view of population structure. Nevertheless, the results are consistent with an interpretation that the local strain diversity of the relapsing fever group species is more limited than that of Lyme borreliosis agents. The intergenic spacer sequences of five *B. miyamotoi* isolates from ticks or mice from

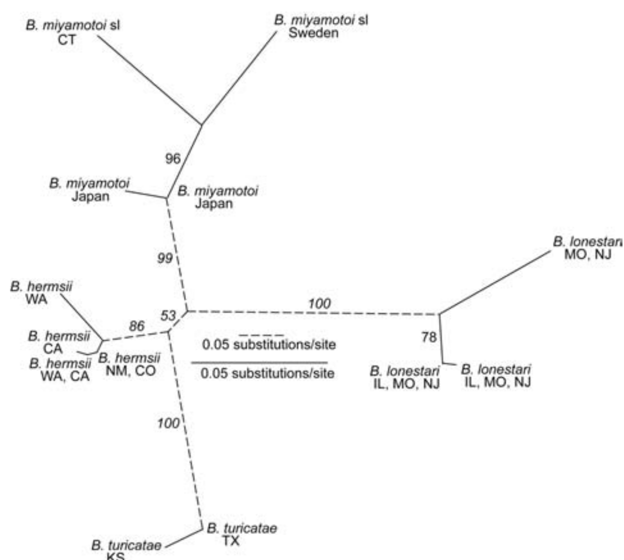

Figure. Unrooted maximum-likelihood phylogram for 16S-23S ribosomal RNA gene intergenic spacer sequences of *Borrelia miyamotoi* s.l., *B. lonestari*, *B. hermsii*, and *B. turicatae*. Maximum likelihood settings for version 4.10b of PAUP* (http://paup.csit.fsu.edu) for equally weighted characters corresponded to Hasegawa-Kishino-Yano model with transition/transversion ratio, nucleotide frequencies, proportion of invariable sites, and gamma distribution shape parameter estimated by maximum likelihood. Support for clades was evaluated by 100 bootstrap replications using full-heuristic search, and values >50% are indicated along branches. Solid and dashed scale bars indicate the number of substitutions per site for the corresponding branches. The geographic origins of the isolates are indicated. The GenBank accession numbers for IGS 16-S rRNA gene intergenic spacer genotype sequences are as follows: *B. miyamotoi* s.l. type 1 (AY363703), type 2 (AY363704), type 3 (AY363705), and type 4 (AY363706); *B. lonestari* type 1 (AY363707), type 2 (AY363708), and type 3 (AY363709); *B. hermsii* type 1 (AY515265), type 2 (AY515266), type 3 (AY515267), and type 4 (AY515269); *B. turicatae* type 1 (AY526494) and type 2 (AY526495). WA, Washington State; CA, California; NM, New Mexico; CO, Colorado; KS, Kansas; TX, Texas; IL, Illinois; MO, Missouri; NJ, New Jersey.

Japan were identical, except for a single nucleotide in one isolate (Figure); the *p66* sequences were identical for each of the five isolates. Four intergenic spacer genotypes were detected from the nine isolates of *B. hermsii* from different regions of the western United States; the three intergenic spacer genotypes that were examined each had a different *p66* allele. Two of the linked intergenic spacer and *p66* genotypes were unique to species from the Rocky Mountain region. The two strains of *B. turicatae* from Texas and Kansas differed in intergenic spacer genotype. *A. americanum* ticks collected in three states yielded three intergenic spacer genotypes from 20 samples positive for *B. lonestari* (Table and Figure). The three intergenic spacer genotypes were each linked to three unique *p66* alleles. Two of the linked genotypes were found at all three loca-

Table. Descriptive statistics for IGS[a] and *p66* loci of three *Borrelia* species

Species	Locus	No. samples	No. variants	Aligned characters			π[b]
				Base pairs	No. gapped	Polymorphisms (%)	
Borrelia miyamotoi s.l.	IGS	33	4	474	15[c]	40 (8.4)	0.058
	p66[d]	19	3	617	9	38 (6.2)	0.042
B. lonestari	IGS	20	3	412	1	14 (3.4)	0.023
	p66[e]	7	3	346	0	5 (1.4)	0.010
B. hermsii	IGS	9	4	665	2	20 (3.0)	0.015
	p66[e]	5	3	516	3	8 (1.6)	0.010

[a]IGS, 16S-23S rRNA gene intergenic spacer region.
[b]π, mean nucleotide diversity at each aligned position.
[c]Excludes an 81-bp indel.
[d]Partial *p66* genes were amplified by nested PCR with outer forward and reverse primers of 5′GATTTTTCTATATTTGGACACAT and 5′AATTTAATCAGATTGTTTAGCTCTA and inner primers of 5′GACACATATCTAAAAAAGCAAACAC and 5′CTAATCCGGTTTTTACGTATATGC and following conditions: 40 cycles of 94°C for 60 s, 55°C for 120 s, and 74°C for 120 s. GenBank accession numbers for *p66* genotypes are the following: *B. miyamotoi* s.l. type 1 (AY363722), type 2 (AY363723), type 3 (AY363724); *B. lonestari* type 1 (AY363689), type 2 (AY363690), type 3 (AY363691); *B. hermsii* type 1 (AF016408), type 2 (AF228028), and type 4 (AF116905).
[e]Analysis of *B. lonestari* and *B. hermsii p66* fragments was limited to 346 of 605 bp and 516 of 608 bp, respectively, which corresponded to the shortest sequences available for all types of the individual species.

tions; one was found in Missouri and New Jersey but not in Illinois.

Conclusions

Samples of the *B. miyamotoi* s.l. showed greater genetic diversity at the intergenic spacer locus than did samples from other genomic groups (Figure). However, even for species with fewer polymorphisms (Table), the intergenic spacer sequences, with or without the *p66* sequences, confirmed the monophyly of strains within each species. This pattern of relationship and the lack of evidence of gene conversion from horizontal gene transfer at this locus, demonstrates that, as for the Lyme borreliosis spirochetes (10), the intergenic spacer region is both sensitive and sufficient for genotyping the relapsing fever group of *Borrelia* species. Sequencing this locus provides a means for further epidemiologic and ecologic studies of the newly discovered *Borrelia* species of hard ticks, as well as of the relapsing fever agents that are reemerging as human pathogens (1). Certain intergenic spacer genotypes of *B. burgdorferi* are associated with certain virulence phenotypes in humans (14). Strain typing by PCR and sequence analysis should also be useful for identifying and characterizing the vertebrate reservoirs of *B. lonestari* and *B. miyamotoi* s.l.

The linkage disequilibrium between the intergenic spacer and *p66* loci indicate that the relapsing fever group species, like Lyme borreliosis spirochetes (10,15), are highly clonal bacteria. Why these two groups of tick-borne spirochetes appear to have different population structures remains to be determined. This variation may be the consequence of differences in pathogenesis between the organisms.

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Salmonella enterica Serotype Uganda Infection in New York City and Chicago[1]

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Outbreaks associated with distinct strains of *Salmonella enterica* serotype Uganda, a rare serotype, occurred in New York City and Chicago during the summer of 2001. Both outbreaks were linked to eating ready-to-eat pork products. This serotype may emerge as a more frequent cause of human infections.

Salmonella enterica serotype Uganda (*S.* Uganda) is rarely isolated from humans in the United States. From 1993 to 2000, a median of 48 human isolates per year (1), and no foodborne disease outbreaks were reported (2). In August 2001, the New York City Department of Health and Mental Hygiene posted an alert on the Epidemic Information Exchange (Epi-X), a national, Web-based communications network for public health investigation and response (3), regarding its investigation of an outbreak of *S.* Uganda infections. Upon detection of a similar cluster in Chicago, the Chicago Department of Public Health notified New York City's health department, and the two agencies discussed methods and results.

The two cities' health departments interviewed case-patients with standardized questionnaires, conducted sanitary inspections of implicated food service establishments, interviewed and tested food workers, and analyzed food and environmental samples. Outbreak isolates were compared by molecular typing with pulsed-field gel electrophoresis (PFGE) with standardized PulseNet protocols (4).

The Outbreaks

New York City

On July 18, 2001, the New York City Department of Health and Mental Hygiene received a complaint of illness from a person who ate at a wedding celebration on July 14; *S.* Uganda was isolated from the stool of another wedding attendee. By early August, a distinct strain of *S.* Uganda had been isolated from 11 New York City residents with illness onsets occurring June 24–August 4 (Figure 1). All 11 case-patients were of Hispanic ethnicity, and 6 of 10 interviewed reported having eaten roast pork from a New York City restaurant in the 3 days before illness onset. Additionally, roast pork from that restaurant had been served at the wedding named in the initial consumer complaint. A sample of leftover roast pork from the wedding was positive for the same strain of *S.* Uganda as the one isolated from patients.

Preparation of the roast pork at the restaurant consisted of seasoning 30–40 lb of raw pork in buckets with salt, pepper, oregano, and garlic. The pork was subsequently cooked in an oven for 2 h at 500°F (260°C), then for 1 to 2 h more at 350°F (177°C). After being cooked, the pork was stored in a hot-holding unit at the front of the restaurant and cut into pieces upon request.

At the time of a sanitary inspection initiated by the consumer complaint, raw pork was held at inadequate temperatures at the restaurant, and thermometers were inadequately used during cooking and hot-holding. Potential sources of cross-contamination, surfaces and wiping cloths, were not properly sanitized. The same *S.* Uganda strain found in patients was isolated from a cooked pork sample collected from the restaurant on July 18. Raw pork sampled at that time was positive for *Salmonella*, but not *S.* Uganda. The restaurant was closed on August 6 and reopened on September 26, after food safety violations were corrected and pork samples were confirmed negative for *Salmonella*.

Chicago

From August 22 to 23, 2001, the Chicago Department of Public Health received reports of four isolates of *S.* Uganda obtained from Chicago residents. By early September, 12 confirmed cases of *S.* Uganda infection had been identified in Chicago with stool collection dates from August 13 to September 6. All 12 patients were of Hispanic ethnicity, and each reported having eaten carnitas (fried pieces of pork) in the 3 days before illness onset. Carnitas were the only food item reported to have been eaten by all the patients. For 10 (83%) patients, the source of the carnitas was a Chicago grocery store. Of the two patients who did not identify that grocery, one had bought carnitas from a mobile lunch truck located outside of his workplace; the source of these carnitas could not be

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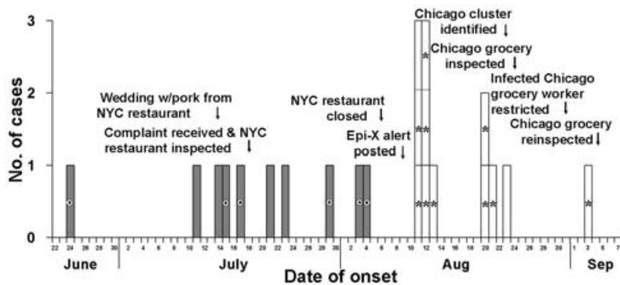

Figure 1. Outbreak-associated *Salmonella enterica* serotype Uganda case-patients by date of illness onset, New York City (NYC) (shaded bars) and Chicago (clear bars), June–September, 2001. Onset date was not given for one NYC case-patient who attended an event at which pork from the implicated restaurant was served. Encircled bullet denotes those who recalled eating pork from the implicated NYC restaurant; asterisk denotes those who recalled eating carnitas from the implicated Chicago grocery.

verified. The other patient was visiting from out of state and could not name the source of the carnitas.

Preparation of carnitas at the Chicago grocery consisted of cutting 90 lbs. of raw pork into 15 cm x 15 cm x 10 cm slabs, frying them in pork lard for 2.5 h, and adding salt and lemon juice 15 min before the end of frying. Cooked carnitas were then chopped, shredded into smaller pieces, and placed in a hot holding tray on top of the meat cooler. Using tongs, a customer would place carnitas into a Styrofoam container, then hand it to a butcher for weighing.

No *Salmonella* was isolated from samples of cooked carnitas, raw pork, the holding tray, or the serving tongs obtained from the Chicago grocery on August 24. *S.* Uganda was isolated from stool from 1 of 10 butchers. He reported having worked while having a mild gastrointestinal illness around August 15 and said he frequently ate carnitas from the Chicago grocery. The infected butcher was restricted from work until two consecutive stool specimens, collected 48 h apart, were negative for *Salmonella*. The workers and grocery manager were advised to avoid contact with food when sick.

The investigation of the Chicago grocery showed that processed meats were being stored at improper temperatures and that potential sources of cross-contamination were not controlled (i.e., cutting boards and utensils were inadequately sanitized, and the same scale was used for raw meats as well as cooked pork). Methods of preventing cross-contamination after cooking were stressed. Upon reinspection, the violations had been corrected.

Comparison of the outbreak isolates by PFGE showed a three-band difference (Figure 2). The sources of the pork implicated in the outbreaks were traced to two separate distributors; a more extensive traceback (i.e., to the packing-plant or farm source) was not conducted.

Conclusions

We report temporally associated outbreaks of *S.* Uganda that likely resulted from eating contaminated ready-to-eat pork products. Including those obtained from outbreak patients in New York City and Chicago, 96 isolates of *S.* Uganda were obtained from human sources in the United States in 2001 (1). This number was the highest reported to the Centers for Disease Control and Prevention in the past decade. This finding is consistent with a previously reported trend toward increased incidence of this serotype; despite an overall downward trend in rates of *Salmonella* infections in the United States, from 1987 to 1997, *S.* Uganda had the eighth highest annual percentage increase in human isolates during this period (5). The outbreaks and these data may signal the emergence of *S.* Uganda as a serotype more frequently associated with human infections in the United States.

Despite the rare occurrence of *S.* Uganda and the temporal association and epidemiologic similarity of the outbreaks, molecular analysis of the outbreak isolates suggested that the New York City and Chicago illnesses were not linked to a common source. The outbreak isolates were also distinguishable from *S.* Uganda clinical isolates posted on the PulseNet Web board in 2001, which indicates a low likelihood that a multijurisdictional common-source *S.* Uganda outbreak had occurred. While this serotype is not among the 10 most common *Salmonella* serotypes found in swine carcasses or raw ground pork (6), it is routinely isolated from swine sources (1). Random sample of *S.* Uganda isolates obtained from swine in 2001 from the National Veterinary Services Laboratory were distinguishable from the outbreak isolates by PFGE. These findings imply heterogeneity in the PFGE patterns of *S.* Uganda isolates and suggest that molecular analysis is a useful tool to assess the relatedness of different strains for outbreak investigations.

Of 357 *Salmonella* foodborne disease outbreaks reported from 1993 to 1997, the vehicle of transmission was classified as pork in 4 (1.1%) outbreaks, a proportion lower than that of beef (3.9%), chicken (1.6%), and turkey (1.6%) (7). Nonetheless, *Salmonella* organisms may be present on an estimated 5%–10% of market hog carcasses after slaughter (8), and the outbreaks described here highlight the potential for *Salmonella* transmission to humans through cooked pork. First, the implicated establishments in New York City and Chicago both cooked large quantities of pork at one time and held the ready-to-eat product in a hot-holding unit; improper holding temperatures could allow growth of any *Salmonella* organisms that survived cooking. Second, food workers cut or weighed the ready-to-eat product before sale at both establishments, and such contact introduces another potential source of contamination. Third, both investigations cited sources of potential

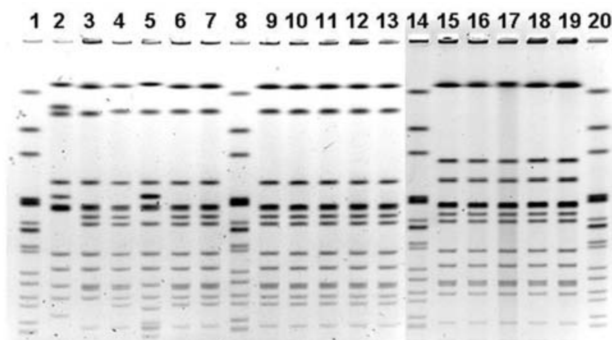

Figure 2. Pulsed-field gel electrophoresis profiles. Lanes 1, 8, 14, and 20, Centers for Disease Control and Prevention standard; lanes 2, 3, and 5, New York City nonoutbreak-associated clinical *Salmonella enterica* serotype Uganda (SU) case isolates; lanes 4, 6, and 9–13, New York City outbreak–associated clinical SU case isolates; lane 7, SU isolate obtained from leftover roast pork from wedding; lanes 15–19, Chicago outbreak-associated clinical SU case isolates.

cross-contamination of cooked product by raw meat. In Chicago, butchers who handled raw meat also served customers, and scales were used to weigh raw meat as well as cooked carnitas. Use of the same knives, cutting boards, or other utensils could have contributed to contamination of the ready-to-eat product in either outbreak. *S.* Uganda was isolated from the stool of one of the Chicago butchers; whether he contributed to disease transmission is unclear. However, infected food workers who do not practice proper hygiene could have played a part in these outbreaks. Previous reports of pork-associated salmonellosis outbreaks have noted the importance of adequately reheating cooked pork if time lapses between preparation and consumption (9,10). Had the pork implicated in the outbreaks been reheated to an internal temperature of 165°F (74°C), the *S.* Uganda infections likely could have been prevented.

A common source was not implicated in the New York City and Chicago outbreaks, but the experience underscores the potential for *Salmonella* transmission through contaminated ready-to-eat pork products, the importance of PFGE in *Salmonella* outbreak investigations even when the serotypes involved are rare, and the utility of the Epi-X system to alert local health departments and facilitate data-sharing.

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Yellow Fever Outbreak, Southern Sudan, 2003

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In May 2003, an outbreak of fatal hemorrhagic fever, caused by yellow fever virus, occurred in southern Sudan. Phylogenetic analysis showed that the virus belonged to the East African genotype, which supports the contention that yellow fever is endemic in East Africa with the potential to cause large outbreaks in humans.

Yellow fever virus (genus *Flavivirus*, family *Flaviviridae*) is a mosquitoborne virus endemic in tropical regions of Africa and South America, where it has been responsible for large epidemics. The virus is maintained in sylvatic transmission cycles involving nonhuman primates but uses humans as the sole vertebrate host in urban epidemics. In South America, the sylvatic vectors belong to the *Haemogogus* and *Sabethes* genera, and the urban vector is *Aedes aegypti*; in Africa, *Aedes* species mosquitoes serve as both sylvatic and urban vectors. The virus causes febrile disease with necrotic hepatitis in humans, and death rates can exceed 50%. Although an effective vaccine is available, the virus remains a major public health threat, particularly in Africa, where vaccination is limited by poverty, civil wars, and the inaccessibility of rural areas where outbreaks of the disease occur. The World Health Organization estimates 200,000 cases of the disease occur annually and 30,000 deaths, but because of underreporting, only a small percentage of these cases are recorded.

The first genetic studies of yellow fever virus identified three topotypes, which corresponded with the virus's geographic distribution in West Africa, Central and East Africa, and South America (1). In a more recent study, which included a larger number of isolates, greater diversity was identified in Africa, with five genotypes designated: Central/East Africa, East Africa, Angola, West Africa I, and West Africa II (2). We report the genetic characterization of yellow fever virus isolates from an outbreak of the disease in southern Sudan in 2003.

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The Study

During the first week of May 2003, the Early Warning and Response Network, established in 1999 in southern Sudan, reported an outbreak of fatal hemorrhagic fever of unknown etiology in the Imatong region of Torit County, which is near the Ugandan border, in a mountainous area covered with tropical rain forest. During the civil unrest in early 2002, many residents were relocated to an internally displaced persons camp in Ikotos County, but in 2003, a number of the residents moved back to the Imatong region. During April and May 2003 suspected cases of hemorrhagic illness were reported, and blood samples collected from Sarianga, Itohom, Lenyleny, Tarafafa, Lofi, and Locomo villages were tested at the Kenya Medical Research Institute (KEMRI), in Nairobi, where yellow fever virus was identified as the causative agent of the outbreak. Aliquots of specimens were submitted to the Special Pathogens Unit at the National Institute for Communicable Diseases in Johannesburg for confirmation of the diagnosis. A report dated June 19, 2003, from the Office of the United Nations Resident and Humanitarian Coordinator for the Sudan described a steady decline in the number of cases occurring during the 4 weeks before the report with a total of 162 suspected cases with 48 deaths in Torit County. However, the report also noted that the recording of suspected cases was inadequate, the figures quoted were an estimate, and final case numbers and deaths were uncertain. The details of the outbreak and laboratory investigations are reported in a separate publication.

Virus isolates were obtained from three patients, two from Locomo village and one from Lofi village. The viral isolations were performed by injecting serum samples from all the patients into suckling mice. RNA was extracted from harvested mouse brain tissue by using Trizol reagent (Gibco BRL, Life Technologies, Gaithersburg, MD), according to the manufacturer's directions. A 670-bp region of the polyprotein gene, which included the 3′ end of the premembrane protein gene, the complete membrane protein gene, and the 5′ end of the envelope protein gene, was amplified by using reverse transcription–polymerase chain reaction (RT-PCR) (2). The nucleotide sequence for each amplicon was determined by using Big Dye Terminator Sequencing Ready Reaction kits with AmpliTaq DNA polymerase FS, as described by the supplier (Applied Biosystems, Warrington, UK). A consensus sequence was established by aligning the sequences obtained from the Sudan 2003 outbreak of yellow fever with sequences obtained from GenBank for yellow fever isolates from previous outbreaks of the virus in Africa

(Table). The sequences from GenBank were selected to represent the five distinct genotypes circulating in Africa (2).

Alignment of the nucleotide sequence data was performed by using DNASIS for Windows version 2.5 (Hitachi Software Engineering America, San Francisco, CA). The phylogenetic analysis was performed on a 572-bp region of the amplicons by using a weighted maximum parsimony method, transversion:transition weighting of 5:1, and Phylogenetic Analysis Using Parsimony (PAUP) software version 4.0b4a for Macintosh (3). Bootstrap confidence intervals were calculated from 100 heuristic search replicates. The tree (Figure) shows that the virus circulating during the recent outbreak in southern Sudan was closely related to an isolate from an outbreak in Kenya in 1993 that belonged to the East African genotype. Sequence divergence was determined by using Molecular Evolutionary Genetics Analysis (MEGA) version 2.1 (4) to calculate the average p-distances or the proportion of pairwise differences within and between groups. The recent Sudan isolates clustered with the East African isolates. A pairwise comparison of nucleotide sequences within the group showed an average distance of 0.111 and 0.179 between the Sudan isolates and an isolate from Kenya (1993) and isolates from Uganda (1948), respectively. No insertions or deletions were found in the Sudan nucleotide sequences compared with the reference strains; however, a number of nucleotide substitutions occurred, most of which were synonymous, and a pairwise comparison of the predicted amino acid sequences showed a high degree of homology. The predicted amino acid sequences were identical for the Kenyan and the Sudan isolates, while the p-distance calculated between the Sudan and Ugandan isolates from 1948 was 0.016. The nucleotide heterogeneity between isolates from East and Central Africa (1.6%–11.6%) supports the concept that different genotypes are circulating and that outbreaks are not the result of imported virus from urban areas (2).

Conclusions

Outbreaks of yellow fever virus have frequently been reported from areas within West Africa since the 18th century, with far fewer outbreaks being identified in East Africa. Serologic evidence for the presence of yellow fever virus in Sudan, Kenya, Uganda, and Tanzania was first reported in 1936 (5). However, not until 1940 was the first epidemic confirmed in East Africa, in central Sudan (5). Sporadic cases were identified annually in East Africa until 1959, when an outbreak was recorded in the Blue Nile region of Sudan and subsequently in the neighboring region of Ethiopia (6). From 1960 to 1962, the largest outbreak to date in Africa occurred in southwest Ethiopia. Additional serologic studies confirmed that yellow fever activity was widespread in Uganda, Somalia, Ethiopia, and Kenya (6). Although two possible cases of yellow fever in Kenya were reported in 1943 (7), not until 1992–1993 was a large outbreak confirmed in the Rift Valley province of Kenya (7,8). Subsequent sporadic isolations of virus have been made in East Africa since then, but no large epidemics were recognized until the outbreak in southern Sudan in 2003. Originally, researchers speculated that outbreaks in East Africa were less frequent and smaller than the large outbreaks recorded in West Africa because they were the result of virus's being introduced into the area at the time of the outbreak. However, the genetic data suggest that yellow fever virus is endemic in East and Central Africa, with outbreaks occurring as a result of favorable environmental conditions (2). The fact that the isolates

Table. History of yellow fever isolates included in the study

Strain	Origin	Year	Source	GenBank no.
SPU160/03/23	Sudan	2003	Human	AY690831
SPU160/03/24	Sudan	2003	Human	AY690832
SPU160/03/25	Sudan	2003	Human	AY690833
14FA	Angola	1971	Human	AF369669
HD 38564	Burkina Faso	1983	Human	AF369670
Serie 227	Ethiopia	1961	Human	AF369674
85-82H	Ivory Coast	1982	Human	U54798
BC 7914	Kenya	1993	Human	AF369676
IB AR 45244	Nigeria	1969	Mosquito	AF369677
H117491	Nigeria	1987	Human	AF369682
Rendu	Senegal	1953	Human	U89338
M 90-5	Sudan	1940	Human	AF369692
M 112-4	Sudan	1940	Human	AF369693
A 709-4-A2	Uganda	1948	Human	AF369694
MR 896	Uganda	1948	Human	U52422
SE 7445	Uganda	1964	Human	AF369695
LSF 4	Zaire	1958	Human	AF369697
SH1464	Senegal	1965	Human	AF369688

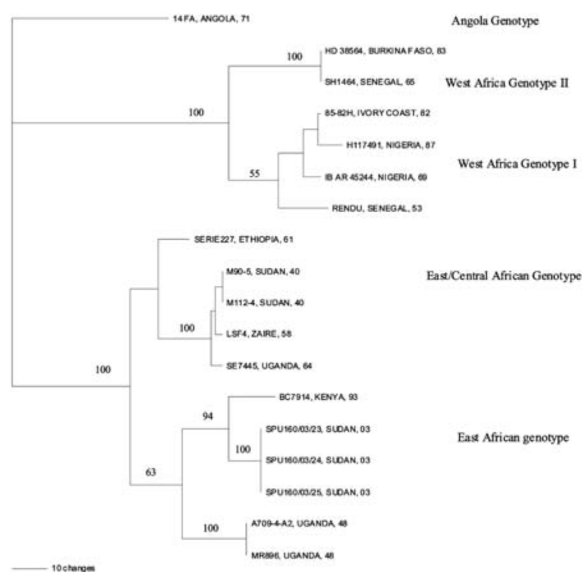

Figure. Phylogenetic tree showing the relationship between yellow fever virus circulating during the outbreak in southern Sudan in 2003 and other isolates from previous outbreaks in Africa, determined by using a 572-bp region of the genome, a weighted parsimony method and Phylogenetic Analysis Using Parsimony (PAUP) software. Node values indicate bootstrap confidence values generated from 100 replicates (heuristic search).

from Sudan were closely related to an isolate obtained 10 years ago in Kenya supports the contention that yellow fever is endemic in East Africa and has the potential to cause large outbreaks when conditions favor transmission to humans.

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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Acinetobacter baumannii in Human Body Louse

Bernard La Scola* and Didier Raoult*

While we were isolating *Bartonella quintana* from body lice, 40 *Acinetobacter baumannii* strains were also isolated and genotyped. One clone was unique and the other was ampicillin susceptible. *A. baumannii* DNA was later detected in 21% of 622 lice collected worldwide. These findings show an *A. baumannii* epidemic in human body lice.

The body louse has been demonstrated to be the vector of three human pathogens: *Rickettsia prowazekii*, the agent of epidemic typhus; *Bartonella quintana*, the agent of trench fever; and *Borrelia recurrentis*, the agent of louseborne recurrent fever (1). While trying to isolate *Bartonella quintana* from body lice of homeless persons in Marseille, we isolated six *Acinetobacter* spp. (2) subsequently identified as *A. baumannii.* They were susceptibile to ampicillin, whereas *Acinetobacter* are almost always resistant to ampicillin in France (3). We further isolated other *A. baumannii* from body lice in Marseille and now have 40 isolates; 21 are susceptible to ampicillin. To investigate the possibility of a clonal diffusion in lice, the *rec*A gene sequence of isolates was determined and compared to that of the collection and strains. To test if the body louse–*A. baumannii* association is observed worldwide, we investigated the presence of *A. baumannii* DNA in a large collection of body lice.

The Study

The 40 body lice–associated *A. baumannii* were obtained during studies of homeless shelters in Marseille (4). The procedure for isolation of these strains has been detailed previously (2). Provisional identification of isolates was based on Gram stain and results of oxidase test and API 20NE identification strip (Biomérieux, Marcy l'Etoile, France). We also tested the 19 strains of *A. baumannii* available at the CIP (Institut Pasteur, Paris, France) and 3 clinical strains isolated in our laboratory during the same period (Table 1). Bacteria were routinely grown at 37°C with 5% CO_2 on Columbia sheep blood agar (Biomerieux). The *rec*A gene amplification was performed with specific primers rA1 (5′-CCTGAATCTTCTGGTAAAAC-3′) and rA2 (5′-GTTTCTGGGCTGCCAAACATTAC-3′), as described previously (5). All sequences were manually edited, and all ambiguous parts were amplified and sequenced again. Amplifying the *rec*A gene allowed unambiguous determination of the sequence of a 336-bp fragment for all isolates. Variation in nucleotides occurred at 10 positions and determined eight genotypes (Table 1), which have been deposited in the GenBank database with the following accession no.: 1, AY274826; 2, AY274827; 3, AY274828; 4, AY274829; 5, AY274830; 6, AY274831; 7, AY274832; 8, AY274833. The translated protein sequences were all identical, except for genotype 2, in which a valine was replaced by an isoleucine at position 68. *rec*A types 1 and 2 were isolated from body louse–associated *A. baumannii*; for the 21 collection strains, seven genotypes were observed. Genotype 2 was unique to body louse–associated *A. baumannii.* Genotype 1 was associated with susceptibility to ampicillin in body louse–associated *A. baumannii* and was common to seven collection strains. However, all collection strains, whatever the genotype, were resistant to ampicillin, even strains of the Unité des Rickettsies that have the same geographic origin as the body louse *A. baumannii*.

We then tested a large collection of body lice for *A. baumannii* DNA. We tested by polymerase chain reaction (PCR) a collection of 622 body lice sampled in France, Burundi, Rwanda, Peru, Algeria, Portugal, and the Netherlands (6). Fifty laboratory lice were used as controls. Detection was performed by amplifying the *rec*A gene with *A. baumannii*–specific primers ACI381F (5′-CACAATGACATTGCAAGCAATTG-3′) and ACI382R (5′-CCAATTTTCATACGAATCTGG-3′) specifically designed for this study. These primers were previously shown not to produce amplicons from *A. calcoaceticus*, *Acinetobacter* genospecies 3, *Acinetobacter* genospecies 13, *A. haemolyticus*, *A. johnsonii*, or *A. lwoffi*. As control for PCR amplification, we used 18Saidg-18Sbi primer pair, which allows amplification of an 18S rRNA gene fragment of arthropods. Consensus forward primer 18Saidg (5′-TCTGGTTGATCCTGCCAGTA-3′) was

Table 1. Types of *rec*A gene sequences[a]

Acinetobacter baumannii strains (n = 62)	*rec*A type	Ampicillin susceptibility
Lice associated (n = 21)	1	Yes
Lice associated (n = 19)	2	No
CIP 70.34, CIP 70.32, UR 121120, CIP 70.8, CIP 70.9, CIP 70.33, UR 73415	1	No
CIP 54.147, CIP 70.28, CIP 103572, UR 37033, CIP 53.77, CIP 70.22, CIP 105742	3	No
CIP 70.24, CIP 68.38	4	No
CIP 70.10, CIP 70.21	5	No
CIP 54.97	6	No
CIP 53.79	7	No
CIP 64.1	8	No

[a]CIP, strains from the Collection de l'Institut Pasteur (Paris, France); UR, clinical strains from Unité des Rickettsies.

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Table 2. Detection of *Acinetobacter baumannii* in body lice from diverse countries by using *rec*A polymerase chain reaction amplification and sequencing

Country	Body lice tested	Detected *A. baumannii* (%)	Sequenced	*recA* type (no.)
France	340	60 (18)	20	1 (18), 2 (2)
Burundi	88	3 (3)	3	1 (3)
Rwanda	45	26 (58)	10	1 (8), 4 (2)
Peru	60	21 (35)	10	1 (3), 3 (5), 4 (2)
Algeria	54	11 (20)	4	4 (4)
Portugal	10	1 (10)	1	1 (1)
the Netherlands	25	8 (32)	2	1 (2)
All tested	622	130 (21)	50	1 (35), 2 (2), 3 (5), 4 (8)

determined after alignment of 18S rRNA sequences of *Drosophila melanogaster* (GenBank accession no. M21017) and *Aedes aegypti* (GenBank accession no. M95126). The consensus reverse primer 18Sbi primer was the one described by DeSalle et al. (7). A total of 130 (21%) body lice were positive for *A. baumannii* (Table 2). None of the 50 laboratory lice was positive. To investigate the genotype association observed among *A. baumannii* strains isolated from Marseille, we sequenced 50 *rec*A amplicons obtained from the lice of different geographic origins (Table 2). Genotypes 1 and 2 were the only ones detected in France; genotype 2 was found in France only. In other parts of the world, genotypes 1, 3, and 4 were observed, with a predominance of genotype 1, similar to the findings in France. Type 4 genotype was the second most common genotype but was absent in European lice. It seems that body louse–associated *A. baumannii* are oligoclonal, and their distribution is different from that of collection strains. Even if genotype 1 is the most common in all cases, genotypes 2 and 4 are overrepresented in body louse–associated strains. However, contrary to culture after body lice decontamination, we cannot rule out that *A. baumannii* infection occurred through external contamination.

Conclusions

The genotype of the 40 *A. baumannii* from Marseille from the body lice of homeless persons are limited to two clones; one is exclusively associated with strains caused by body lice, and the other is associated with ampicillin susceptibility in body louse–associated strains. This finding shows an *A. baumannii* epidemic in body lice. *A. baumannii* is mainly implicated in cases of hospital-acquired infections but has also been reported as a cause of severe community-acquired infections, including pneumonia, endocarditis, and meningitis, mostly in persons who are alcoholics (8). While ingesting only blood from humans, the louse has a sterile midgut, and the presence of bacteria is likely caused by the louse's ingesting contaminated blood (2). Moreover, previous studies have shown that *A. baumannii* is not a common skin-associated *Acinetobacter* in Europe, unlike in tropical areas, since it is found on the skin of <1.5% of healthy persons (9). Our results indicate that association of *A. baumannii* with body lice is likely caused by undiagnosed transient *A. baumannii* bacteremia in patients harboring body lice; however, because the frequency of skin association of *A. baumannii* in the homeless subpopulation is unknown, contamination from body lice cannot be ruled out. Relatively low-virulence flora, such as *Staphylococcus epidermidis* or diptheroids, may be destroyed by leukocytes, antibody, and complement in the blood meal, whereas the more virulent bacteria, such as *A. baumannii*, could survive because they resist the defense mechanism of the blood meal and those of the body lice. However, we never isolated *S. aureus* from lice, which is a virulent bacterium and known to be a common skin commensal agent. From preliminary work, we have observed that body lice may be infected by several bacterial species (L. Houamdi, unpub. data) and that the occurrence of body louse–transmitted disease occurs because causative bacteria (*B. quintana*, *R. prowazekii*, and *B. recurrentis*) induce relapsing bacteremia rather than specifically adapting to body lice (10). Finally, if our hypothesis of *A. baumannii* bacteremia in patients harboring body lice is true, their clinical manifestations in homeless persons remain to be determined.

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Dr. Raoult is director of the Unité des Rickettsies, the national reference center for rickettsiosis and WHO collaborative center. His work focuses on the study of emerging and reemerging bacteria and arthropodborne diseases.

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Fluoroquinolone Resistance in *Salmonella enterica* Serotype Choleraesuis, Taiwan, 2000–2003

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Salmonella enterica serotype Choleraesuis is a highly invasive pathogen that infects humans and causes systemic infections that require antimicrobial therapy. Surveillance in Taiwan showed that fluoroquinolone resistance in *S.* Choleraesuis markedly increased from 2000 to 2003, reaching approximately 70% in 2003.

Of the more than 2,000 nontyphoid *Salmonella* serotypes, *Salmonella enterica* serotype Choleraesuis is extremely invasive and usually associated with bacteremia in humans. Before 1999, this serotype was susceptible to fluoroquinolones (1). Since 2000, we have recorded rapidly increasing resistance to ciprofloxacin in *S.* Choleraesuis isolated from both human and swine sources in Taiwan (2). This finding is a cause for concern because fluoroquinolones are first-line drugs to treat systemic, nontyphoid salmonellosis. We conducted a prospective, laboratory-based surveillance study of fluoroquinolone resistance in *S.* Choleraesuis isolated from humans in four major teaching hospitals across Taiwan from 2000 to 2003.

The Study

Chang Gung Memorial Hospital in Keelung is a 1,000-bed hospital. Chang Gung Memorial Hospital in Taoyuan, which includes Chang Gung Children's Hospital, is located in Taoyuan and has a capacity of 4,000 (which includes an additional 500 beds for Chang Gung Children's Hospital). Chang Gung Memorial Hospital in Kaohsiung, in southern metropolitan Taiwan, is a 2,000-bed hospital. Chang Gung Memorial Hospital in Chiayi, which was open for service in December 2001, is an 800-bed hospital. All four are teaching hospitals affiliated with Chang Gung University in Taoyuan, Taiwan. The geographic locations of these hospitals are shown in Figure 1. All *S.* Choleraesuis isolates were cultured and identified according to standard methods (3) in the clinical microbiologic laboratories of the four hospitals. No major changes were made in the policy concerning identification of *Salmonella* during the study years. *Salmonella* isolates were first checked with O antisera (Becton Dickinson and Co., Franklin Lakes, NJ) for their serogroups by the slide agglutination method. Because it causes invasive infections, *S.* Choleraesuis was specifically identified if a negative result was found in the citrate utilization test for any isolate identified as serogroup C1 *Salmonella*. H antiserum has been used to verify the accuracy of this procedure in our previous studies (1,2). The susceptibility to ciprofloxacin of the isolates was investigated by a standard disk-diffusion method (4). Susceptible or resistant isolates were defined according to the criteria suggested by the National Committee for Clinical Laboratory Standards (4); isolates in the intermediate category were deemed as resistant in this study.

The annual isolate number of *S.* Choleraesuis among the four Chang Gung Memorial Hospitals and the resistance to ciprofloxacin from 2000 to 2003 are summarized in Figure 2. An apparent growing trend of resistance was observed. The overall resistance rate of the isolates collected from the four hospitals increased from 32.3% in 2000 to 56.5% in 2001, 61.8% in 2002, and 71.8% in 2003 ($p < 0.00001$ by chi-square test). Ciprofloxacin resistance rate was generally lower in 2000; however, such resistance increased to >50% in Keelung, Taoyuan, and the newly opened Chiayi Chang Gung Memorial Hospitals in 2001 to 2002. The resistance rate remained constantly high in 2003. The highest rate was found in clinical isolates from

Figure 1. Geographic position of the four hospitals in Taiwan where *Salmonella enterica* Choleraesuis isolates were collected and surveyed for their susceptibility to ciprofloxacin, 2000–2003.

*Chang Gung Children's Hospital, Taoyuan, Taiwan; †Chang Gung Memorial Hospital, Taoyuan, Taiwan; and ‡Chang Gung Memorial Hospital, Kaohsiung, Taiwan

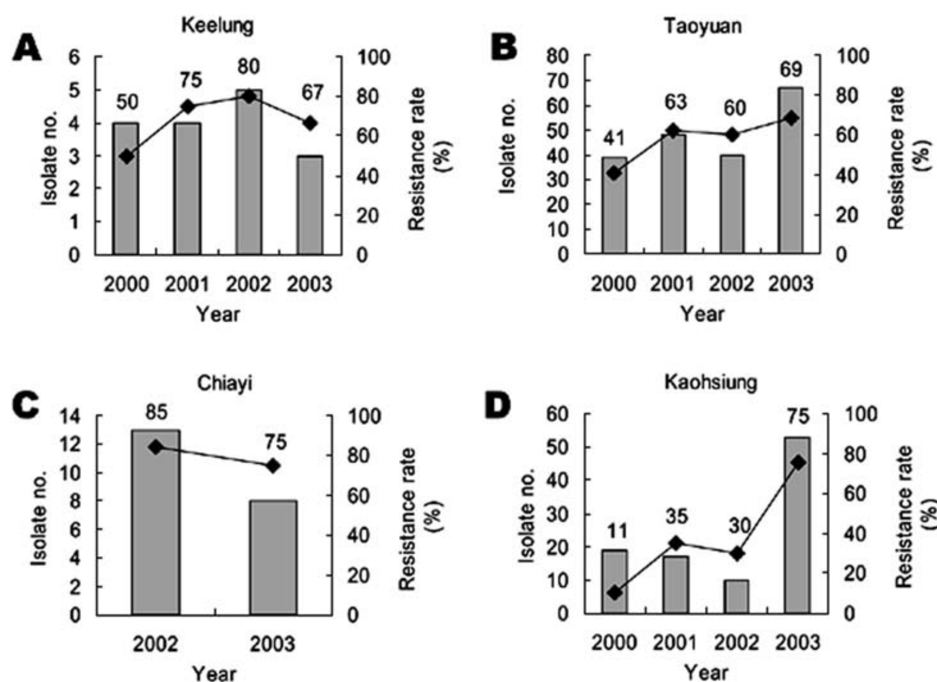

Figure 2. The annual isolate number (in bars) and the rate of ciprofloxacin resistance (in lines) among the isolates from the four hospitals in which *Salmonella enterica* Choleraesuis isolates were collected and surveyed for their susceptibility to ciprofloxacin, 2000–2003.

Chang Gung Memorial Hospital in Chiayi: 11 (85%) of 13 isolates were resistant in 2002. Although approximately 30% of the *S.* Choleraesuis isolates were resistant to ciprofloxacin in Chang Gung Memorial Hospital, Kaohsiung, before 2002, an upsurge to 76% was detected in this hospital in 2003.

Conclusions

S. Choleraesuis is an infrequent serotype of *Salmonella* and has been isolated from humans in the United States (5). The annual reported number of *S.* Choleraesuis isolates from humans was approximately 80 from 1990 to 1996. The number decreased gradually thereafter, with an annual isolate number of 49 in 1997, 36 in 1998, 34 in 1999, and 15 in 2000 (5).

However, this highly invasive serotype is of particular concern in Taiwan; it was the second most common *Salmonella* isolate found in humans in two independent epidemiologic surveys (6,7). The reason for the difference between Taiwan and United States is an issue of interest. *S.* Choleraesuis is highly host-adapted to pigs. We have found that *S.* Choleraesuis isolates from humans and swine had the same or similar DNA fingerprints, which suggests that human infections were derived from pigs (2). Such infection likely arose as a result of the contamination of a food or water source. Eating pig offal by the local population likely contributed to the high prevalence of *S.* Choleraesuis infection in some areas of Taiwan.

Results of this surveillance study reflect the current status of fluoroquinolone resistance in *S.* Choleraesuis in Taiwan. The four hospitals surveyed in this study are located in different regions across Taiwan, the surveillance is prospective and longitudinal in design, and the overall isolate number examined is large enough to draw a conclusion. Minor geographic variation in the rate of ciprofloxacin resistance was observed. Among the four hospitals surveyed, Chang Gung Memorial Hospital in Kaohsiung is the only one located in a metropolitan area. Sanitation in a metropolitan area is better than that in the rural or country areas. The spread of resistant clones, therefore, may be slower in this area, as reflected by the lower number of *S.* Choleraesuis infections treated in Kaohsiung Chang Gung Memorial Hospital compared to those treated in Linko Chang Gung Memorial Hospital. Moreover, most pig farms are not located in large cities. These factors might explain why the spread of resistance in Kaohsiung was slower than in other regions before 2002. In contrast, isolates from Chiayi Chang Gung Memorial Hospital, which is located in the underdeveloped, southwestern coastal region of Taiwan, showed the highest rate of resistance to ciprofloxacin in 2002. The up-to-date data obtained in 2003 confirmed a wide dissemination of ciprofloxacin-resistant *S.* Choleraesuis across Taiwan.

We have found that all of the resistant strains carried mutations that give rise to the substitution of phenylalanine for serine at position 83 and asparagine for aspartic acid at position 87 in GyrA (2). In addition, mutations leading to an amino acid change from serine to isoleucine at position 80 in ParC were demonstrated in the ciprofloxacin-resistant *S.* Choleraesuis isolates (8,9). The emergence of fluoroquinolone resistance in *S.* Choleraesuis was mainly due to the dissemination of an endemic, resistant clone (2,9).

The emergence of fluoroquinolone resistance in this highly invasive nontyphoid *Salmonella* serotype represents a serious threat to public health and clinical medicine. Most of the ciprofloxacin-resistant *S.* Choleraesuis were also multidrug resistant to the conventional antimicrobial agents (1,2,6,10). This resistance indicates that extended-spectrum cephalosporins become the only reliable agent in the treatment of systemic infections caused by *S.* Choleraesuis. However, in 2002, we isolated for the first time from a patient with sepsis, a strain of *S.* Choleraesuis that was simultaneously resistant to ceftriaxone and ciprofloxacin (8). The ceftriaxone resistance gene, bla_{CMY-2}, of this isolate was found on a potentially transmissible plasmid (8); thus, the spread of such resistance phenotype may be unavoidable in the future. In view of the severe situation, restricted use of antimicrobial agents in both humans and domestic animals as well as actively monitoring *S.* Choleraesuis isolates for antimicrobial drug resistance should be reinforced.

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The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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Barriers to Creutzfeldt-Jakob Disease Autopsies, California

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Creutzfeldt-Jakob disease (CJD) surveillance relies on autopsy and neuropathologic evaluation. The 1990–2000 CJD autopsy rate in California was 21%. Most neurologists were comfortable diagnosing CJD (83%), but few pathologists felt comfortable diagnosing CJD (35%) or performing autopsy (29%). Addressing obstacles to autopsy is necessary to improve CJD surveillance.

Transmissible spongiform encephalopathies (TSEs) are rare, progressively fatal, neurodegenerative illnesses. Human TSEs include classic Creutzfeldt-Jakob disease (CJD) and the recently described variant CJD associated with eating bovine spongiform encephalopathy–infected cattle products in Europe (1). The recent identification of bovine spongiform encephalopathy in the United States underscores the importance of maintaining enhanced surveillance to monitor for the possible occurrence of variant CJD in this country (2,3).

In California, CJD is not reportable. Since 1999, the California CJD Surveillance Project of the California Emerging Infections Program, a collaboration of the California Department of Health Services and the U.S. Centers for Disease Control and Prevention, has conducted enhanced surveillance for classic and variant CJD. Methods include review of state mortality data and follow-up investigation of CJD-related deaths that occur in persons <55 years of age, since >98% of cases of variant CJD in the United Kingdom have occurred in this age group. As part of this enhanced surveillance, medical records for 33 deceased California residents <55 years old from 1996 through 2003 have been investigated with criteria for CJD developed by the World Health Organization and Centers for Disease Control and Prevention; none met the criteria for variant CJD.

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Currently, pathologic review of brain tissue obtained by biopsy or autopsy is the only means of confirming a diagnosis of CJD. Autopsy remains the preferred method for obtaining tissue, as brain biopsy can result in serious complications (e.g., brain hemorrhage or abscess formation) and may not yield adequate amounts of tissue for analysis. The main role of brain biopsy is to exclude other, potentially treatable conditions (4).

In this article, we describe results from analysis of California mortality data from 1990 through 2000. We also summarize responses generated from a statewide survey of neurologists and pathologists regarding the challenges to diagnosing CJD and variant CJD, including obtaining autopsy in suspected cases.

The Study

Data from the 1990–2000 Death Public Use File (underlying cause of death only) and 1990–1999 Multiple Cause-of-Death Data (underlying or contributing causes of death) were obtained from the Center for Health Statistics, California Department of Health Services (5). Deaths among California residents with an International Classification of Diseases, 9th Revision, code 046.1 or 10th Revision, code A81.0 listed anywhere on the death record were included in our analysis. Both data files included report of autopsy as a variable, with the exception of the Multiple Cause-of-Death Data for 1997 to 1999, when autopsy performance was not recorded. Statistical analysis was performed by using SAS software (SAS Institute, Cary, NC).

From July to December 2002, questionnaires regarding experience with diagnosing CJD were sent to 1,241 California neurologists identified as members of the American Academy of Neurology and 574 pathologists identified as members of the California Society of Pathologists and the American Association of Neuropathologists. Approval was obtained from the Committee for the Protection of Human Subjects of the State of California.

Review of mortality data identified 263 CJD-related deaths in California from 1990 through 2000. Of these, 244 were identified from the 1990–1999 Multiple Cause-of-Death Data, and an additional 19 deaths were identified from the 1990–2000 Death Public Use File. A total of 42 (16%) cases identified by the Multiple Cause-of-Death Data were not detected in the Death Public Use File. Overall, 26 (10%) of the 263 CJD-related deaths were in persons <55 years of age. Only two deaths occurred in persons <30 years of age. The overall autopsy rate, which for 1997 to 2000 only includes autopsies performed on persons for whom CJD was recorded as the underlying cause of death, was 53 (21%) of 251 persons: 11 (44%) of 25 persons <55 years of age, and 42 (19%) of 226 persons

≥55 years of age. For two deaths, autopsy performance was not recorded.

Of 1,241 questionnaires mailed to neurologists, 428 (34%) were completed, including 310 (25%) from respondents involved in patient care. Responses regarding the neurologists' experience with diagnosing CJD and performing autopsy are summarized in Tables 1 and 2. Most neurologists (83%, 255/307) felt comfortable clinically recognizing classic CJD. More than one third (36%, 74/207) had not considered arranging for autopsy in their CJD patients, although most reported access to histopathologic services (75%, 223/297). The most commonly cited barrier to obtaining autopsy was family reluctance to give consent (79%, 192/242).

Of 574 questionnaires mailed to pathologists, 284 (49%) were completed. Tables 1 and 2 summarize the responses. Thirty-five percent (96/273) and 15% (40/274) of pathologists were comfortable recognizing the neuropathologic features of classic CJD and variant CJD, respectively. Infection control concerns (77%, 143/185), lack of experience (62%, 69/111), and institutional limitations (53%, 111/210) were cited as major obstacles to autopsy performance, and less than half of respondents reported that confirming the diagnosis of CJD (47%, 92/197) or ruling out variant CJD (45%, 87/193) was an important reason to pursue autopsy.

Conclusions

Our analysis suggests that autopsy rates for CJD in California are low. The results of our surveys, which attempted to discern the reasons for this low rate, imply that both neurologists and pathologists have similar perceptions of the value of obtaining histopathologic evaluation for CJD but for different reasons. Most neurologists appeared to be comfortable clinically diagnosing CJD, with more than one third reporting they had never considered pursuing autopsy for CJD cases. In contrast, pathologists appeared to be less comfortable making a histopathologic diagnosis, indicating that autopsy performance was limited by infection control concerns, lack of experience with CJD cases, and institutional restrictions.

Our results have some limitations. Approximately 10% of CJD cases may have atypical signs and symptoms that can obscure the diagnosis. To the extent that these cases are misdiagnosed and not autopsied, they could contribute to overestimation of the autopsy rate. On the other hand, death certificate analysis can be an insensitive indicator of the true rate of autopsy, and autopsy performance information was unavailable for 1997 to 2000 from the Multiple Cause-of-Death Data. Both factors could lead to possible underestimation of the true autopsy rate. Given that some CJD cases will have had confirmatory brain biopsy or strongly suggestive clinical features and diagnostic studies, the autopsy rates cited may apply mostly to patients for whom a satisfactory antemortem diagnosis could not be made. Interpreting survey results is limited by the low response rate; neurologists and pathologists who are experienced in diagnosing CJD may be more likely to respond, which would introduce bias.

The public health benefits of performing autopsy on patients with suspected CJD should not be underestimated. Autopsy and histopathologic analysis remain important ways to confirm a diagnosis of CJD and help define the usual occurrence of subtypes of classic CJD, thereby facil-

Table 1. Knowledge and experience of California neurologists, pathologists, and neuropathologists in diagnosing Creutzfeldt-Jakob disease (CJD)

Characteristic	Neurologists n/N (%)	Pathologists n/N (%)	Neuropathologists n/N (%)
Have evaluated a case of CJD	212/310 (68)	56/259 (22)	18/33 (55)
Median no. (range) of CJD cases evaluated	3 (0–30)	2 (0–30)	10 (0–50)
Type of practice			
Private practice/private hospital	144/308 (47)	122/278 (44)	8/33 (25)
Outpatient HMO[a]/managed care	55/308 (18)	–	–
Community hospital/clinic	1/308 (<1)	68/278 (24)	4/33 (12)
University affiliated	82/308 (27)	37/278 (13)	10/33 (30)
Veterans hospital	13/308 (4)	3/278 (1)	1/33 (3)
County medical examiner or coroner	–	7/278 (3)	2/33 (6)
Other	15/308 (5)	41/278 (15)	5/33 (15)
Can recognize the clinical or pathologic features of classic CJD	255/307 (83)	96/273 (35)	25/28 (89)
Can recognize the clinical or pathologic features of variant CJD	120/305 (39)	40/274 (15)	18/28 (64)
Have not considered arranging for an autopsy for CJD patients under their care	74/207 (36)	–	–
Pathology group available at facility to perform autopsy on suspect CJD cases	223/297 (75)	74/259 (29)	17/28 (61)
Pathology group available at facility to confirm diagnosis of suspect CJD with histopathologic analysis	223/297 (75)	91/254 (36)	18/27 (67)

[a]HMO, health maintenance organization.

Table 2. Perceptions of California neurologists, pathologists, and neuropathologists regarding performance of autopsy in Creutzfeldt-Jakob disease (CJD)

Characteristic	Neurologists n/N (%)	Pathologists n/N (%)	Neuropathologists n/N (%)
Important reasons to obtain autopsy for CJD patients			
Autopsy is needed to confirm CJD diagnosis	–	92/197 (47)	11/21 (52)
Autopsy is needed to rule out variant CJD or other TSE[a] forms	168/231 (73)	87/193 (45)	12/20 (60)
Barriers to performing autopsy and histopathologic analysis for CJD			
Clinicians do not feel autopsy is required for diagnosis	94/221 (43)	72/198 (36)	7/21 (33)
Facilities not able/willing to perform autopsies on CJD patients	75/234 (32)	111/210 (53)	8/22 (36)
Families are reluctant to give consent for autopsy	192/242 (79)	57/202 (28)	6/22 (27)
Cost of autopsy is a concern to patient's family	113/234 (48)	34/202 (17)	8/20 (40)
Cost of autopsy is a concern to hospital/institution	78/234 (34)	40/199 (20)	8/21 (38)
Infection control is a concern regarding autopsy	102/235 (44)	143/185 (77)	9/11 (82)
Facilities are inadequate to perform autopsy	–	24/185 (13)	5/11 (45)
Infection control is a concern regarding histopathologic evaluation	–	62/111 (56)	4/8 (50)
No available pathologists experienced in recognizing histopathologic features of CJD	–	69/111 (62)	1/8 (13)

[a]TSE, transmissible spongiform encephalopathy.

itating the recognition of emerging TSEs (1,6,7). Autopsy rates for nonforensic deaths have declined dramatically during the past 40 years, with national hospital rates currently <5%, possibly resulting in missed diagnoses of the actual cause of death in 8% to 25% of cases (8–11). The reasons for the decline are multifaceted and include escalating cost of autopsy borne by hospitals and county medical examiners, lack of direct reimbursement, fear of litigation, and increasing reliance on modern technology to determine a diagnosis antemortem (10).

Our survey results suggest that infection control concerns play a role in low autopsy rates for CJD, whether because of fears about the risk of acquiring CJD from handling contaminated tissue or because of liability considerations at the institutional level. More realistically, brain autopsy can be performed safely as long as CJD-specific infection control guidelines are strictly followed (12–13). Nonetheless, concerns about potentially acquiring CJD through autopsy procedures should be acknowledged and recognized as an opportunity to address proper infection control techniques.

Enhancing surveillance for variant CJD and other emerging prion diseases will require educating neurologists and pathologists, addressing the perceived obstacles to obtaining autopsy, and encouraging the use of available resources that provide expertise and technical assistance in evaluating CJD. For example, brain tissue can be submitted to the National Prion Disease Pathology Surveillance Center (NPDPSC) in Cleveland, Ohio, for free state-of-the-art diagnostic testing (14). The availability of a national center of expertise may facilitate obtaining tissue evaluation; since the inception of NPDPSC, the number of referrals to the facility has more than doubled, from 104 in 1997 to 265 in 2002, and the number of TSE cases confirmed from those referrals increased from 60 in 1997 to 151 in 2002 (14). Regional academic institutions, such as the University of California, San Francisco, Memory and Aging Center, can also provide expertise and assistance with diagnostic testing. Such resources are vital to maintaining vigilance for cases of CJD and potentially emerging human TSEs, such as variant CJD or possibly a human form of chronic wasting disease in the United States.

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Leishmaniasis in Refugee and Local Pakistani Populations

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The epidemiology of anthroponotic cutaneous leishmaniasis was investigated in northwest Pakistan. Results suggested similar patterns of endemicity in both Afghan refugee and Pakistani populations and highlighted risk factors and household clustering of disease.

In Central Asia, anthroponotic cutaneous leishmaniasis (ACL) is commonly caused by *Leishmania tropica* and characterized by large, chronic, and disfiguring skin ulcers, which often cause severe social stigma. Because ACL is transmitted anthroponotically (i.e., from human to human) by sandflies, the infection can spread rapidly in concentrated populations, particularly under poor housing conditions, i.e., overcrowding or lack of protection from bloodsucking insects. In Afghanistan, the incidence of endemic but sporadic ACL has dramatically increased during decades of civil war, because of the associated deterioration of the infrastructure and migration (1–3). Less is known about the current distribution of the disease in neighboring Pakistan, where it has always been widespread but considered "patchy" and nonendemic (4). Recently, however, local authorities and nongovernmental health providers have reported an increasing number of ACL cases in Afghan refugee camps (5,6), which causes concern about the potential spread of the disease among the population and local Pakistani villagers. Therefore, a large-scale epidemiologic study was conducted throughout northwest Pakistan to investigate this issue.

The Study

From December 2002 to March 2003, a study was conducted in 48 Afghan refugee camps and 19 neighboring villages in Balochistan and North-West Frontier Province (NWFP), Pakistan. Refugee camps were selected on the basis of past and present ACL cases reported by healthcare providers. Villages within 1 km of selected camps were included in the survey; if multiple villages were within 1 km of a camp, one with reported ACL cases was randomly selected, although this method may have introduced selection bias. The goal of the study was to estimate the prevalence of ACL in Afghan refugee camps and neighboring Pakistani villages, as well as determine whether refugee camps could be the source of the anecdotal rise in ACL cases in neighboring villages. In each site, 40 households were sampled along east-west and north-south perpendicular transects. Every head of household was interviewed with a standard questionnaire. If a family reported cases of ACL, an interviewer who had been trained in clinical ACL diagnosis asked to inspect the lesions. Because of logistic constraints, no parasitologic confirmation was performed, but lesions caused by organisms other than *Leishmania* are rare, and our previous studies have shown that specificity of our clinical diagnosis is 73%–76% (5).

The study included 21,046 persons in 48 refugee camps and 7,305 persons in 19 neighboring villages. Overall, 650 persons (2.3%) had ACL lesions only, 1,236 (4.4%) had ACL scars only, and 38 persons had both ACL lesions and scars. Of those with active ACL, the mean lesion number was 2.1 (range 1–16), and the mean lesion duration (to survey date) was 5.1 months (range 0.7–50 months). Using maximum likelihood methods (7), we estimated the average annual force of infection of ACL to be 0.01 per year (10 cases/1,000 persons per year) during the past 6 years.

In refugee camps, the prevalence of ACL lesions was 2.7%, and prevalence of scars was 4.2%. In neighboring Pakistani villages, the prevalence of ACL lesions was 1.7%, and prevalence of scars was 4.9%. Lesion prevalence increased with age more markedly among local Pakistanis than Afghan refugees until children were 5–6 years of age; then the prevalence of lesions decreased among Pakistanis and was lower than in the Afghan refugee population for all remaining age groups (Figure). These age trends suggest past infection and resultant immunity. Had the disease been introduced more recently, the risk of ACL would not be expected to be related to age, since everyone would be susceptible to infection (8). However, the low prevalence of scars relative to the number of active lesions, especially among adults, suggests that the disease has been endemic in the region for a short period of time and that transmission may be characterized by a prolonged epidemic similar to that found in Kabul (2,4).

To examine the association with potential risk factors and to take clustering of persons within households into account, univariate odds ratios (OR) were estimated by logistic regression with robust standard errors. We used backward stepwise multiple logistic regression to identify significant explanatory risk factors while controlling for other variables. Spatial clustering of ACL was investigated

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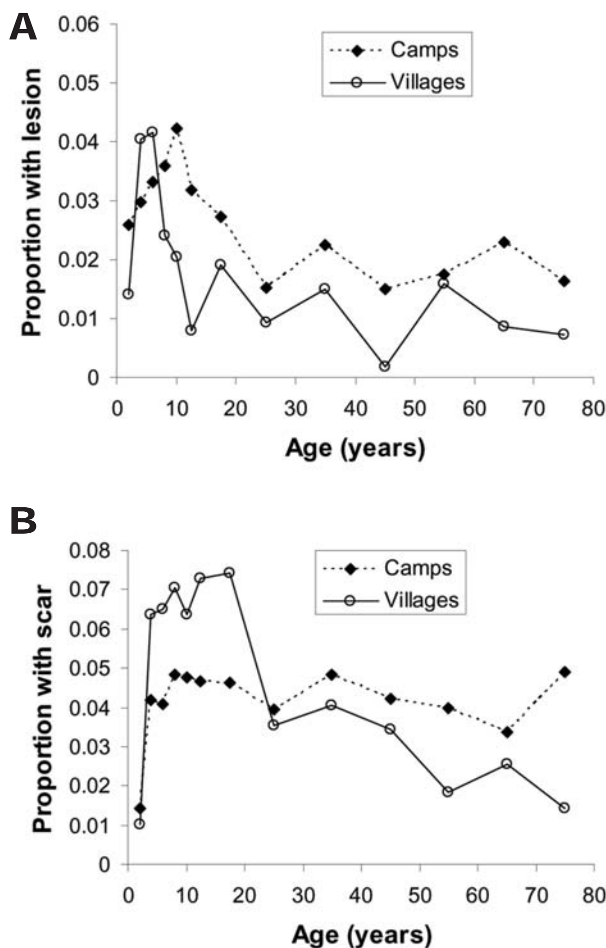

Figure. A) Proportion of unscarred population with active lesions by age and settlement type. B) Proportion of population with scar by age and settlement type.

at the household and village levels. The degree of within-household clustering was calculated by using a random-effects model fitted to a logistic regression to account for the nonindependence of persons within households. The analysis was conducted using STATA 8 (Stata Corporation, College Station, TX). The nonparametric Mantel correlation statistic with Mantel 2 (Queensland University of Technology, Brisbane, Australia) was used to assess spatial correlation in prevalence between settlements by investigating the relationship between differences in lesion prevalence and geographic distances.

The univariate analysis showed that an increased risk of ACL lesion was associated with years lived in camp or village, a family member visiting Afghanistan in the last 12 months, household members with ACL lesions, household members having ACL scars, age group, household with stone walls, crowding in the household (i.e., the number of people per room), having cows in a compound, and having dogs in a compound (Table 1). The same variables were significantly associated with the risk of having an ACL scar, with the exception of a family member's having visited Afghanistan in last 12 months. Use of a mosquito net was associated with an increased risk of having a scar. Multivariate analysis showed that younger age, as well as ACL lesions in other household members, increased the risk of an ACL lesion (Table 2). Increased risk of an ACL scar was associated with younger age, living in a refugee camp, and scars in other household members. No significant interactions were detected among the other variables included in the analysis. Finally, after age, sex, and household factors were adjusted for, the random effects model found evidence for significant household clustering of active ACL cases: $\rho = 0.54$ (95% confidence interval [CI] 0.49–0.59, $p < 0.0001$). ACL scars clustered in households to an even greater degree: $\rho = 0.62$ (95% CI 0.59–0.65, $p < 0.05$). The prevalence of ACL lesions showed a marked variation (0%–21.9%) between Afghan refugee camps and neighboring Pakistani villages. However, analysis using the Mantel correlogram indicated no spatial structuring of ACL between neighboring villages, which emphasizes the highly focal distribution of ACL transmission at the village level and corroborating significant household clustering of ACL.

Conclusion

The analysis of putative risk factors for ACL indicated that living in a stone house reduced the risk, whereas the presence of cows and dogs increased it (Table 2). Although dogs have been found infected with *L. tropica* (9), they are probably not leishmaniasis reservoirs, as transmission of *L. tropica* is thought to be anthroponotic (2). Instead, dogs and other domestic animals represent an additional feeding source for sandflies, which increases contact between vectors and humans. Improved housing protects against vector-borne diseases, since it reduces human-vector exposure. Reported household use of a mosquito net was associated with increased risk of ACL scar, which may reflect the practice of selling insecticide-treated nets at highly subsidized prices to refugee households with active ACL.

Although parasite identification was not carried out in this study, that *L. tropica* is the etiologic agent seems probable because it causes most leishmaniasis cases in Central Asia (5,10), and transmission is characterized by clustering of cases and higher risk among children. Our data indicate that parasite transmission is autochthonous in surveyed sites, although highly heterogeneous between sites. Observed childhood-acquired immunity indicates that not all cases are imported from Afghanistan, as has been suggested (5). Consequently, continual and vigilant surveillance is required to monitor the epidemiology of ACL in the region. The mass return of *Leishmania*-infect-

Table 1. Unadjusted odds ratios for variables associated with the risk of anthroponotic cutaneous leishmaniasis lesion and scar[a]

Variable	Lesion [OR (95% CI)]	Scar [OR (95% CI)]
Village[b]	χ^2 = 540, df = 66, p < 0.001	χ^2 = 786, df = 66, p < 0.001
Refugee camp (compared to local village)	1.540 (1.16–2.06), p = 0.003	0.82 (0.62–1.09), p = 19
Nationality (Afghan compared to Pakistani)	1.050 (0.78–1.38), p = 0.720	0.940 (0.70–1.26), p = 0.680
Years lived in camp/village	1.010 (1.01–1.02), p < 0.001	1.002 (0.99–1.01), p = 0.510
Family member visited Afghanistan in last 12 mo.	1.740 (1.37–2.20), p < 0.001	1.690 (1.35–2.11), p < 0.001
Lesion prevalence in other household members	1.120 (1.11–1.13), p < 0.001	1.040 (1.03–1.06), p < 0.001
Scar prevalence in other household members	1.030 (1.02–1.03), p < 0.001	1.090 (1.08–1.10), p < 0.001
Sex (female compared to male)	1.010 (0.89–1.15), p = 0.770	1.050 (0.95–1.16), p = 0.310
Age group (compared to 0–4 y)		
5–19 y	1.090 (0.87–1.37), p = 0.450	1.750 (1.44–2.13), p < 0.001
≥20 y	0.560 (0.43–0.71), p < 0.001	1.080 (1.07–1.59), p = 0.007
Type of wall (compared to mud)		
Brick	0.940 (0.33–2.68), p = 0.920	0.640 (0.31–1.31), p = 0.220
Stone	0.530 (0.32–0.88), p = 0.010	0.480 (0.30–0.77), p = 0.002
Other	2.000 (0.76–5.21), p = 0.150	1.160 (0.59–2.31), p = 0.650
Type of ceiling (compared to cloth)		
Concrete	0.690 (0.26–1.79), p = 0.450	1.090 (0.44–2.71), p = 0.850
Wood (beam)	1.430 (0.65–3.17), p = 0.370	1.590 (0.73–3.48), p = 0.240
Wood (thatched)	0.460 (0.16–1.27), p = 0.140	1.770 (0.70–4.49), p = 0.230
Other	0.850 (0.30–2.41), p = 0.760	1.180 (0.47–2.99), p = 0.710
Rooms/person	0.200 (0.07–0.56), p = 0.002	0.430 (0.21–0.91), p = 0.020
Cows in compound (yes/no)	1.420 (1.22–1.65), p < 0.001	1.510 (1.34–1.69), p < 0.001
Dogs (yes/no)	1.660 (1.42–1.94), p < 0.001	1.310 (1.16–1.48), p < 0.001
Meshed windows (% windows covered)	1.260 (0.51–3.13), p = 0.610	0.720 (0.32–1.61), p = 0.420
Use mosquito net	1.180 (0.83–1.67), p = 0.350	1.560 (1.19–2.05), p = 0.001
Treated mosquito net	0.760 (0.32–1.78), p = 0.530	0.740 (0.42–1.32), p = 0.320

[a]OR, odds ratio; CI, confidence interval; df, degrees of freedom.
[b]The overall significance of this categorical variable is shown rather than the 67 different survey locations.

ed refugees to urban areas in Afghanistan poses a particular risk, since housing is often poor, and living conditions are crowded. Including ACL prevention measures in Afghanistan's basic package of health services (e.g., supplying insecticide-treated nets to areas at high risk) should be considered to prevent the spread of disease through previously ACL-free urban areas.

Current ACL interventions in the study areas in Pakistan are funded by the United Nations High Commissioner for Refugees (UNHCR) and mainly focus on Afghan refugees. Free diagnosis on the basis of clinical symptoms, analysis of specimens by microscope, and treatment with antimony are provided for all patients attending basic health units in refugee camps, and insecticide-treated nets are sold at highly subsidized prices to refugees with active ACL. The local population is not a focus of the program, since resources are limited. Insecticide-treated net users in local villages either make their own nets or acquire them through "leakage" of nets intended for Afghan refugees or at communities across the

Table 2. Adjusted odds ratios associated with risk of anthroponotic cutaneous leishmaniasis lesion and scar, based on multiple logistic regression model, using village as a random effect variable

Variable	Adjusted OR (95% CI)[a]
Lesion	
Age group (compared to 0–4 y)	
5–19 y	1.17 (0.90–1.52) p = 0.320
≥20 y	0.48 (0.35–0.65) p < 0.001
Lesion prevalence in other household members	1.10 (1.09–1.11) p < 0.001
Scar	
Age group (compared to 0–4 y)	
5–19 y	2.52 (1.93–3.29) p < 0.001
≥20 y	1.99 (1.48–2.69) p < 0.001
Refugee camp (compared to local village)	1.48 (1.03–2.14) p = 0.040
Scar prevalence in other household members	1.08 (1.07–1.11) p < 0.001

[a]OR, odds ratio; CI, confidence interval.

border in Afghanistan. Long-term control of ACL transmission in Pakistan will require extending diagnostic and treatment services and building up a program to sell insecticide-treated nets to the local population. With the ongoing reduction in UNHCR funding and anticipated phasing-out of support to refugee health care at the end of 2005, the population will depend on the Pakistan Ministry of Health to deliver these much needed services.

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Botulism Type E Outbreak Associated with Eating a Beached Whale, Alaska

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We report an outbreak of botulism that occurred in July 2002 in a group of 12 Alaskan Yu'pik Eskimos who ate blubber and skin from a beached beluga whale. Botulism death rates among Alaska Natives have declined in the last 20 years, yet incidence has increased.

The incidence of botulism in Alaska is among the highest in the world, and all cases of foodborne botulism in Alaska have been associated with eating traditional Alaska Native foods, including "fermented" foods, dried foods, seal oil, and muktuk (skin and a thin pinkish blubber layer immediately underneath the skin) from marine mammals (1,2). Botulism toxins are divided into seven types; intoxication with toxin type E is exclusively associated with eating aquatic animals. Most cases of botulism in Alaska are caused by toxin type E.

On July 12, 2002, two residents of a Yup'ik village in western Alaska found a carcass of a beached beluga whale that appeared to have died sometime that spring. They collected the tail fluke for consumption, cut it into pieces, and put the pieces in sealable plastic bags. Portions were refrigerated and distributed to family and friends. From July 13 to July 15, a total of 14 persons ate some of the raw muktuk. On July 17, a physician from western Alaska reported three suspected cases of botulism from this village; all patients had eaten the muktuk. The Alaska Department of Health and Social Services began an immediate investigation to ensure proper treatment of the ill persons, identify and interview other persons exposed to the implicated food, and obtain clinical and food samples for laboratory testing.

The Study

We sought to identify and interview every person who ate the muktuk; all exposed persons identified were evaluated by a physician or nurse practitioner for signs or symptoms of botulism. Exposed persons with signs or symptoms compatible with foodborne botulism were referred to the regional hospital for further evaluation and treatment, if necessary. A case of foodborne botulism was defined as a clinically compatible illness (Figure) with symmetric descending flaccid paralysis of motor and autonomic nerves in a person who had eaten the muktuk. Serum, stool, and gastric specimens from case-patients and leftover muktuk were collected and submitted to the Centers for Disease Control and Prevention (CDC) National Botulism Surveillance and Reference Laboratory for botulinum toxin detection, using the standard mouse bioassay (1).

Of 14 persons identified who ate the muktuk, 8 (57%) had illness that met the case definition. Three of the eight patients were male; the median age was 73 years (range 13–83 years). The median incubation period was 24 hours (range 12–72 hours). Signs and symptoms are shown in the Table. Five (63%) patients were hospitalized, four (50%) received types AB and E antitoxin a median of 30 hours (range 24–60 hours) after symptom onset, two (25%) required mechanical ventilation, and all survived. Both persons who required mechanical ventilation received antitoxin. Attending clinicians decided not to give antitoxin to four of the patients with milder illness because of the potential risk for adverse side effects from horse serum antitoxin.

Three stool, three gastric fluid, and seven serum samples from the eight patients and seven samples of whale muktuk were tested for botulinum toxin. The mean sample collection interval for serum was 3 days after exposure (range 1–5 days); for stool and gastric fluid, the mean interval was 4 days after exposure (range 3–6 days). The diagnostic laboratory received all laboratory specimens on July 26, and results were reported on August 1. Type E

Clinical findings
- Cranial nerve palsies
- Intact mental status despite groggy appearance
- Normal body temperature
- Normal sensory nerve and deep tendon reflex examination findings
- Symmetrically descending flaccid paralysis of motor and autonomic nerves
    - Diminishing forced vital capacity (check every 4 hours)
    - Ileus (atonic)
    - Weakness (e.g., new inability to stand up from sitting position or walk a flight of stairs)
    - Postural hypotension
    - Urinary retention (diagnose with a postvoid residual test)

Diagnostic test findings
- Normal cerebrospinal fluid values
- Specific electromyography findings, including
    - Normal motor conduction velocities
    - Normal sensory nerve amplitudes and latencies
    - Decreased evoked muscle action potential
    - Facilitation after rapid repetitive nerve stimulation
- Standard mouse bioassay positive for toxin from clinical specimens and/or suspected food; requires up to 4 days for final results

Figure. Clinical and laboratory findings of foodborne botulism.

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Table. Signs and symptoms of eight case-patients from a botulism outbreak associated with eating a beached whale, western Alaska, July 2002

Sequelae	No. (%)
Gastrointestinal symptoms	
Abdominal pain	5 (63)
Constipation	5 (63)
Diarrhea	4 (50)
Nausea or vomiting	7 (88)
Neurologic symptoms	
Blurred vision	5 (63)
Diplopia	1 (13)
Dry mouth	7 (88)
Dysphagia	6 (75)
Dysarthria	4 (50)
Shortness of breath	5 (63)
Other symptoms	
Throat pain	3 (38)
Dizziness	6 (75)
Neurologic signs	
Hoarse voice	5 (63)
Ptosis	2 (25)
Pupils fixed and dilated	5 (63)
Urinary retention	1 (13)
Weakness	8 (100)
Other signs	
Bradycardia[a]	4 (50)
Hypotension[b]	6 (75)

[a]Heart rate <60 beats per minute.
[b]Systolic blood pressure <100 mm Hg.

toxin was detected in a stool sample from one patient. This stool sample was collected on day 5 after exposure and received for testing 7 days later. All other clinical specimens were negative for botulinum toxin. All seven samples of muktuk were positive for type E botulinum toxin.

Conclusions

An outbreak of botulism type E affected 8 of 14 Alaska Natives who ate muktuk harvested from a dead beached whale found on the remote Alaska Bering Sea littoral. Illness was promptly diagnosed and antitoxin administered. Although the median serum, stool, and gastric fluid sample collection times were within 4 days of illness onset, and all muktuk samples tested positive for toxin type E, only 1 of the 13 clinical samples from case-patients yielded positive results for toxin with the standard mouse bioassay. Both the limited sensitivity of the mouse bioassay for botulinum toxin detection in clinical specimens, as seen in this outbreak, and the fact that the test requires up to 4 days for final results demonstrate that clinicians should not wait for laboratory confirmation to make diagnostic and clinical treatment decisions.

Almost half of the cases of all types of foodborne botulism in the United States occur in Alaska, which has 0.2% of the national population. From 1990 to 2000, a total of 97 cases of botulism type E were reported in the United States; 91 (92%) occurred in Alaska. Alaska Native death rates from botulism have dropped during recent decades. Arctic explorers and whalers described deaths of entire Alaska Native families who ate whale meat (3). Before 1961, the botulism case-fatality rate among Alaska Natives was nearly 50%; from 1967 to 1974, it declined to 9% (4). From 1990 to 2000, the case-fatality rate averaged 3%, lower than that of the other 49 states. This reduction is due to several factors. First, public health efforts have educated the population and clinicians serving it about prevention, signs, symptoms, and the need for immediate treatment of botulism. Second, immediate evacuation of rural patients to modern regional hospitals, often by small aircraft, is routinely practiced. Third, trivalent botulinum antitoxin (anti-A, B, and E) is stocked in most rural hospitals so it is immediately available for treatment when clinically indicated (5).

Type E toxin is responsible for >85% of all botulism cases in Alaska because many traditional Alaska Native foods, including salmon heads, whale blubber, seal flesh and oil, and fish eggs are prepared by fermentation under conditions that may favor germination and vegetation of toxin type E–producing *Clostridium botulinum* (4,6). Eating blubber from whale carcasses as described in this outbreak is in accordance with tradition; however, storing blubber in airtight sealable plastic bags, which can create an anaerobic environment, is a modern development. The use of airtight containers to store and ferment traditional foods is theorized to be at least partly responsible for the increase in incidence of foodborne botulism in Alaska from 1970 to 1997 (5).

In his 1963 review of botulism type E, Dolman suggested a logical solution to the problem of botulism in the Arctic when he stated that "Public health educational efforts based on a proper understanding of the dangers involved can do much to reduce them by advocating relevant sanitary precautions. Besides, merely drawing attention to these hazards will accelerate the march of acculturation and thus eventually render [traditional Alaska Native foods] unpopular (6)." Since that time, however, anthropologic and paleontologic studies have found negative health effects associated with rapid cultural transformation (7), and studies have found that rapid changes from subsistence diets to affluent (Western) diets have been associated with increased incidence of coronary heart disease, obesity, diabetes mellitus, and cancer among Alaska Natives (8–11). In addition, eating traditional foods can form part of the cultural identity of groups that are in cultural transition and therefore may be perpetuated as a link to the past (2). To that end, state and federal public health officials have invested considerable effort in promoting intake of traditional foods among Alaska Natives, while defining safer methods for food storage and preparation (5,12).

Because botulism may have nonspecific symptoms (e.g., abdominal cramping, diarrhea, vomiting), rapid diagnosis can be difficult, even in Alaska, where the index of suspicion is comparatively elevated. However, some specific signs and symptoms (e.g., descending paralysis) may be detectable early on and are virtually diagnostic (Figure). The clinical signs and symptoms of botulism from toxin types A, B, and E are similar; however, a clinical comparison in the United States from 1975 to 1988 showed that patients with illness from type E toxin have slightly shorter incubation periods and less frequently required intubation (39% with type E, 52% with type B, and 67% with type A) (13). Additional current reports on botulism, including the epidemiology, diagnosis, therapy, outbreak response, and reference laboratories have been recently published (1,14).

In conclusion, clinicians confronted with a suspected botulism case should review the clinical diagnostic criteria and not rely on the mouse bioassay for toxin detection to guide clinical decision-making. In addition, although public health efforts and clinical improvements have drastically reduced botulism death rates among Alaska Natives, the average annual incidence of foodborne botulism in Alaska since 1970 is substantially higher than it was before 1970, possibly, in part, because of modern influences, such as availability of plastic, sealable containers. Finally, because of the numerous beneficial health (not to mention cultural) effects of traditional food consumption, we recommend that Alaska Native leaders continue to promote traditional food consumption among their people, while educating them about the potential hazards of improper storage and preparation (12).

Dr. McLaughlin is a medical epidemiologist with the Alaska Section of Epidemiology. His epidemiologic interests include infectious disease epidemiology, coronary heart disease among Alaska Natives, and electronic disease reporting surveillance systems.

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Toronto Emergency Medical Services and SARS

To the Editor: The first appearance of severe acute respiratory syndrome (SARS) in China in November 2002 led to a worldwide epidemic by March 2003. On February 21, 2003, an index case of SARS, which led to 224 cases and 38 deaths, was diagnosed in Toronto. On March 14, four cases of atypical pneumonia in Toronto were epidemiologically linked to the SARS outbreak in China. On March 26, the Ontario Ministry of Health declared a provincewide medical state of emergency, which was lifted on May 17 when the SARS outbreak was thought to be over. However, 7 days later, several more cases of SARS were discovered in four Toronto hospitals, which caused a resurgence of the intensive precautionary measures throughout the healthcare sector. When the state of emergency was lifted on July 2, 2003, a total of 224 people in Toronto had been officially diagnosed with SARS, and 38 had died.

The SARS outbreak strained Toronto Emergency Medical Services (EMS), which worked 40 stations evenly divided among the city's four quadrants. Annually, Toronto EMS transports >140,000 patients to 17 acute-care hospitals, which makes it the largest and busiest municipal EMS in Canada. During the outbreak, Toronto EMS's 850 paramedics had 1,166 potential SARS exposures; 436 were placed in a 10-day home quarantine, which meant being isolated from those persons within the home, continuously wearing an N95 respirator, and taking their temperature twice a day. SARS-like illnesses developed in 62 paramedics, and suspected or probable SARS requiring hospitalization developed in 4 others. On March 26, almost all of the frontline staff of the city's northeast quadrant were sent home because of possible SARS exposure at a Toronto hospital (1). On May 22, when the outbreak's second phase began, >200 paramedics had contact with patients with SARS and were quarantined. These events seriously affected EMS and their staff.

Even before the SARS emergency was declared in Ontario, Toronto EMS was aware of a serious respiratory disease in the community. Because of an increase in "atypical pneumonia" cases, an advisory had been sent to all paramedics warning them to wear respirators, gowns, gloves, and goggles with all respiratory patients. The advisory was recalled in favor of the Provincial Directive; the Provincial Directive was also changed when SARS reemerged in May. While properly fitting and supplying 850 paramedics with respirators took several months, no paramedics became ill with SARS after these requirements were initiated, even without fit-testing all the respirators.

Although cleaning the emergency vehicles was a potential concern, the only important change was substituting the usual disinfectant of 3% hydrogen peroxide with virucidal effect in 10 minutes to a disinfectant of 7% activated hydrogen peroxide with virucidal effect in 5 minutes. Otherwise, normal procedures were followed and emergency vehicles were cleaned on their regular rotational basis.

During the outbreak, the EMS Healthcare Divisional Operations Centre became the emergency operations center for Toronto EMS. It had been designed to coordinate Toronto's operational response with other municipal and provincial health services. During this time, the province also created its own emergency operations center, to which representatives from both health services reported.

Within days of the provincial emergency, Toronto EMS, in conjunction with Toronto police and fire services, created the medical support unit that operated as an internal public health department for all paramedics and was responsible for their direction, education, support, and screening. If needed, paramedics were placed under work or home quarantine or precautionary symptom surveillance on the basis of their exposure history, symptoms, and treatment in an emergency department or SARS clinic if needed. The medical support unit used protocols developed by a base hospital medical director who, together with EMS staff, reviewed each paramedic's chart daily to make appropriate follow-up decisions. The medical support unit was a vital component in protecting the paramedics' health and welfare.

To sustain the optimal functioning of Toronto EMS, its headquarters was closed to frontline staff for the duration of the outbreak. All personnel had to be screened for SARS-like symptoms before entering, and all paramedics had to check themselves for signs and symptoms of a SARS-like illness before reporting for duty. Anyone with SARS-like symptoms had to report to the medical support unit and stop working in an EMS capacity.

To control the spread of SARS, the provincial government placed all interfacility transfers under the control of Toronto EMS through the creation of the Provincial Transfer Authorization Centre on March 29. Since then, the Provincial Transfer Authorization Centre has been responsible for ensuring that all nonemergency transfers are medically cleared to prevent patients with contagious diseases from being taken to a facility that is unprepared to receive them. The Provincial Transfer Authorization Centre now processes >1,200 requests daily and was an important factor in containing SARS.

Several lessons were learned from the SARS outbreak. First, an emergency plan must be in place before an outbreak occurs. Second, the ability to

communicate quickly and easily with provincial and municipal health authorities was needed to ensure that the most up-to-date information concerning the outbreak was available. The intergovernmental relationships necessary for such rapid communication should be established in advance. Third, accurate and timely communication with frontline staff members is the best way to minimize their fears. Finally, personal protective equipment procedures should be maintained until assurance that the exposure risk is negligible. The SARS outbreak is unlikely an isolated occurrence; therefore, sound advance planning on the basis of experience will increase the ability to protect both EMS staff and the public in the future.

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SARS during Pregnancy, United States

To the Editor: Two of eight persons with laboratory-confirmed severe acute respiratory syndrome– associated coronavirus (SARS-CoV) infection in the United States during 2003 were pregnant women. Robertson et al. (1) reported data describing one pregnant patient who recovered and delivered a healthy infant. We report data concerning the second patient, with follow-up 1 month after the child's birth.

The patient, a healthy, 38-year-old woman in the 7th week of pregnancy, traveled with her husband to Hong Kong. From March 1 to March 6, 2003, they stayed at the Hong Kong hotel where it is believed a physician from China spread SARS-CoV to several guests. These guests were the index case-patients for subsequent outbreaks in Hong Kong, Vietnam, Singapore, and Toronto, Canada (2). The woman and her husband returned to the United States on March 6; the husband had onset of SARS illness on March 13. On March 19, the patient had onset of an illness with fever (temperature 37.8–40°C), muscle aches, chills, headache, runny nose, productive cough, wheezing, and shortness of breath. A chest radiograph showed a diffuse infiltrate in the left lung. The patient was hospitalized for 9 days and given broad-spectrum antimicrobial drugs. She recovered from her illness, and enzyme immunoassay and immunofluorescent assays conducted on serum samples on days 28 and 64 after illness onset were positive for antibodies to SARS-CoV.

The patient had an uneventful pregnancy until the last trimester, when her blood glucose levels were elevated. Early spontaneous rupture of membranes initiated preterm labor, and a cesarean section was performed at 36 weeks' gestation because of fetal distress. A 5-pound, 7-ounce, healthy boy was delivered without complications. Apgar scores were 7 at 1 minute and 8 at 5 minutes. The newborn had no illness, abnormalities, or congenital malformations. Serum samples from the patient at delivery were positive for antibodies to SARS-CoV, but cord blood and placenta samples were negative. Breast milk samples on postpartum days 12 and 30 were also negative for SARS-CoV antibodies. Blood, stool, and nasopharyngeal swab samples from the patient and cord-blood samples showed no viral RNA by reverse transcription–polymerase chain reaction. Stool samples from the newborn, collected on days 12 and 30 after delivery, were also negative for viral RNA.

Although other countries have reported cases of severe illness and poor outcome associated with SARS-CoV infection during pregnancy (3–5), neither of the two pregnant SARS case-patients in the United States had serious adverse outcomes. The presence of antibodies to SARS-CoV in breast milk might be influenced by the time of infection in relation to gestation. Robertson et al. (1) reported that antibodies to SARS-CoV were detected in the breast milk of a patient who was infected at 19 weeks' gestation; however, the patient in this case was infected at 7 weeks' gestation, and antibodies to the virus were not detected in her breast milk. No reports have indicated vertical transmission of SARS-CoV, a finding that is supported by our data. However, too few cases have been studied to clearly define the risks and provide guidance for treating pregnant women infected with SARS CoV.

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Eosinophilic Pleural Effusion in Gnathostomiasis

To the Editor: Moore et al. reported a case series of patients infected with imported gnathostomiasis who had typical intermittent, migratory skin manifestations or peripheral blood eosinophilia or both, as well as undiagnosed eosinophilia with nonspecific symptoms (1). We would like to add some comments based on a recent patient treated in Marseille, France.

In April 2003, a 66-year-old man living in Marseille indicated a history of fever for 8 days. He had returned from Vietnam 1 month earlier, where he had stayed for 4 weeks in the Ho Chi Minh City area and 2 days in the Mekong Delta area. He was well during his trip, and reported no arthropod bites except from mosquitoes. He had no direct skin contact with river water. Dietary intake included local dishes with rice, fish, pork, shrimp, and chicken. His symptoms started 3 weeks after he returned to Marseille and included fever (temperature 38°C), asthenia, chills (1 day), moderate dyspnea during exercise, transient bilateral pain of the testes, and an episode of hemospermia. He was referred by his family doctor to the Infectious and Tropical Diseases Unit, North Hospital.

On admission, the patient's temperature was 38°C. Physical examination of the patient, including the testes, was normal except for a systolic heart murmur (preexisting and known to the patient), and clinical signs of left pleural effusion. The effusion was subsequently confirmed by chest x-ray, which also showed a discrete diffuse bilateral lung infiltrate. Results of routine laboratory tests conducted on blood samples were normal except for an elevated eosinophil count of 5.2 10^9/L. Blood smears for plasmodia and microfilaremia were negative. Urologic examination, including echography and prostate-specific antigen, showed no abnormalities except a prostatic adenoma (preexisting and known to the patient). No eggs or parasites were detected by microscopic examination in stools or in urine, although both sedimented and centrifuged urine specimens were studied and filtration techniques were used. After transthoracic aspiration of 100 mL of pleural effusion, cytologic examination showed an eosinophil count of 5,800/L without parasites. Bacterial culture, including mycobacteria, was negative. On day 4, the patient was afebrile and was discharged. On admission, results of a first set of examinations involving reactivities to schistosomiasis, paragonimiasis, strongyloidiasis, cysticercosis, trichinosis, gnathostomiasis, filariasis, and toxocariasis were negative.

One month later, the eosinophil count of our patient had decreased to 1.8 x 10^9/L. He was afebrile, and his only complaint was asthenia. A new set of serologic examinations was conducted. The Western blot assay for gnathostomiasis conducted at the Swiss Tropical Institute (Socinstrasse 57, CH-4002, Basel, Switzerland) was positive, showing immunoglobulin G reactivity to four specific bands including the 24-kDa band, considered pathognomonic for the diagnosis of *Gnathostoma* infection (1). The seroconversion confirmed the diagnosis of gnathostomiasis. All other serologic tests remained negative, except an increase of antibodies against *Acanthocheilonema vitae* used as antigen for unspecific serologic screening for filariasis (Laboratoire Marcel Merieux, Lyon, France). After a 21-day course of albendazole and a single dose of ivermectin, the eosinophil count of our patient decreased to 0.8 x 10^9/L.

Three aspects of gnathostomiasis as an emerging imported disease can complement the findings of Moore et al. (1). First, the clinical findings in our case are very unusual. Hemospermia is often benign with predominant causes including prostatic and seminal vesicle disease. Infections, including mainly schistosomiasis and tuberculosis, have been associated with these symptoms (2). Although our patient had a prostatic adenoma, this is the first time that hemospermia has been associated with gnathostomiasis. Because of the anxiety hemospermia caused, this symptom was the main reason that our patient consulted our center. Secondly, eosinophilic pleural effusion is also unusual in gnathosthosmiasis. Although reported as a potential cause in reference books (3), a Medline search (key words: gnathostomiasis and eosinophilia and pleural effusion or pleuritis or lung) disclosed only two references to pleural effusion as the main symptom of gnathostomiasis (4,5).

The eosinophilic pleural and pulmonary response may be elicited by the larvae of helminths carried hematogenously into lungs and pleura in an aberrant fashion (3). The last point we stress is that, as shown by Moore et al. (1), patients returning from disease-endemic areas, mainly Southeast Asia and Central and South America, should be tested systematically for gnathostomiasis. Although some patients show a typical cutaneous form of gnathostomiasis associated with eosinophilia (6,7), most atypical forms are probably underdiagnosed, and severe neurologic involvement may occur if treatment is not given (1). However, until recently specific serologic tests for gnathostomiasis were available only in Asia, mainly in Thailand and Japan. Some laboratories in Europe currently provide testing for gnathostomiasis, which would be a valuable aid in evaluating patients returning from the tropics.

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Methicillin-resistant *Staphylococcus aureus*, Pakistan, 1996–2003

To the Editor: This letter is written in response to the article titled "Co-trimoxazole-sensitive, methicillin-resistant *Staphylococcus aureus*, Israel, 1988–1997" (1). We found the authors' findings most interesting. As the authors pointed out, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become a major problem worldwide. The problem is not restricted to industrialized countries. The last decade has seen an alarming increase in MRSA infections in Pakistani hospitals (2). Pakistan's Armed Forces Institute of Pathology provides laboratory services to a 1,500-bed tertiary-care hospital in Rawalpindi and is the main reference laboratory in northern Pakistan. According to our computerized database, the frequency of MRSA among all nosocomial isolates of *S. aureus* increased from 39% (212/543) in 1996 to 51% (516/1,018) in 2003 ($p < 0.0001$). Most of the isolates were obtained from pus and pus swab specimens (153 in 1996 and 394 in 2003), while the rest were obtained from blood (20 in 1996 and 37 in 2003), intravenous catheter tips and surgical drainage tubes (14 in 1996 and 31 in 2003), various body fluids (9 in 1996 and 19 in 2003), respiratory secretions (8 in 1996 and 18 in 2003), tissue (4 in 1996, 9 in 2003), throat swabs (2 in 1996, 6 in 2003), and urine (2 in 1996, 5 in 2003).

During the last 7 years, resistance in MRSA isolates has steadily increased to most of the antimicrobial drugs such as gentamicin (69% in 1996 and 88% in 2003), ciprofloxacin (87% in 1996 and 94% in 2003), clindamycin (60% in 1996 and 70% in 2003), and rifampicin (20% in 1996 and 60% in 2003). However, resistance to co-trimoxazole and doxycycline has decreased. In 1996, 15% (32/212) of our MRSA isolates were susceptible to co-trimoxazole, whereas in the first 9 months of 2003, 43% (222/516) of the isolates were susceptible ($p < 0.0001$). Similarly, susceptibility to doxycycline increased from 34% in 1996 to 49% in 2003 ($p = 0.0005$). Antimicrobial drug susceptibility of the isolates was tested by the modified Kirby-Bauer technique and results were interpreted according to the National Committee for Clinical Laboratory Standards criteria (3). Methicillin resistance was tested by using 1 µg oxacillin disks (Oxoid, Basingstoke, Hampshire, UK) on Mueller-Hinton agar containing 4% sodium chloride. Plates were incubated at 35°C for 24 hours.

We agree with Bishara et al. (1) that the increase in susceptibility is likely due to decreased use of these antimicrobial drugs for staphylococcal infections in clinical practice. The use of co-trimoxazole in our hospital decreased from 48 daily doses per 1,000 hospital days in 1996 to 35 daily doses in 2003, while use of doxycycline decreased from 12 daily doses per 1,000 hospital days in 1996 to 9 daily doses in 2003 (4). These antimicrobial drugs offer an inexpen-

sive alternative to glycopeptides for the treatment of MRSA infections. Data from the United States and Europe have shown that vancomycin-intermediate *S. aureus* isolates also remain susceptible to some of the conventional antimicrobial drugs, including co-trimoxazole (5). If their efficacy in vivo is validated by clinical trials, use of these conventional drugs would not only reduce the load on overstretched health care budgets but reduce the use of vancomycin, therefore decreasing the risk of isolates continuing to develop vancomycin resistance.

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Borrelia valaisiana in Cerebrospinal Fluid

To the Editor: Lyme borreliosis is the most common tickborne human disease in the Northern Hemisphere. The incidence of the disease is not the same throughout Europe; in southern Europe, the incidence ranges from 43% in Croatia to 1.1% in Greece. Suspected borreliosis cases have been reported in Greece, none were confirmed. *Ixodes ricinus*, the principal tick vector of *Borrelia burgdorferi* in Europe, is found in northern Greece. A low prevalence of *B. burgdorferi* antibodies was found in healthy persons in Greece (1,2); a frequency of 7.3% was found in arthritis patients (1), while a frequency of 16.9% was found in patients with neurologic disorders (E. Diza, unpub. data).

Polymerase chain reaction (PCR) has been used to detect *B. burgdorferi* DNA in humans and to determine genospecies (3). Isolates found in the United States have constituted a homogeneous group. In Europe, five different genospecies from the original *B. burgdorferi*, now called *burgdorferi sensu lato* complex, have been described: *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii, B. valaisiana*, and *B. lusitaniae*. Pathogenicity for humans remains uncertain for *B. valaisiana* and *B. lusitaniae* (4).

Neuroborreliosis, the most serious manifestation of disseminated Lyme disease, has become the most frequently recognized arthropodborne infection of the nervous system in the United States and Europe. *B. garinii, B. afzelii*, and *B. burgdorferi sensu stricto* are confirmed causes of neuroborreliosis (5); however, *B. valaisiana* has not been isolated from cerebrspinal fluid (CSF) until this report.

We report the genetic detection of *B. valaisiana* in the CSF of a 61-year-old man with a history of spastic paraparesis, which is strong clinical evidence of advanced neuroborreliosis. Symptoms, mainly difficulty in walking, began approximately 10 years earlier, with a slow progressive course of neuroborreliosis. His medical history showed an unidentified sexually transmitted disease in 1982, an undefined episode of arthritis in the lower limbs in 1990, and a nonspecific rash in the genitals in 1995. The patient lived in South Africa from 1961 to 1997 and visited Thassos Island in northern Greece every year. The neurologic examination demonstrated an intense pyramidal spasticity in the lower limbs and moderate weakness (Medical Research Council grade 3) of the proximal muscles. Serial magnetic resonance imaging (MRI) of the brain showed small hyperintensities in the periventricular area on T2-weighted images; MRI of the spinal cord showed no abnormalities. Multiple sclerosis, B12 deficiency, human T-cell lymphotrophic virus-1 infection, structural inflammatory lesions of the spinal cord, motor neuron disease, and hereditary spastic paraplegia have been excluded. The patient was treated occasionally with intravenous penicillin G, as well as with corticosteroids, but no clinical improvement was achieved. Venereal disease reaction level was negative and all tests for syphilis in CSF were negative.

DNA was extracted from CSF, and a region of the chromosomal flagellin gene of *B. burgdorferi* was amplified by nested PCR (3). *B. afzelii* (VS461) DNA was used as a positive control. All precautions were taken to avoid contamination. The amplified PCR product was sequenced, and the sequence (Th1) was deposited in GenBank with the accession no. AY270021. Phylogenetic analysis showed that strain Th1 was clustering with strains belonging to *B. valaisiana* genomic group. Specifically, a nucleotide difference

of 0.38% was observed among Th1 and isolates Ku10 and To76 (accession no. AYO83505 and AYO83504, respectively), which belong to *B. valaisiana* genomic group and were isolated from *ricinus* in Sweden (6). A genetic difference of 0.77% was observed between Th1 and *B. valaisiana* strain Tr29 (accession no. ABO91805) isolated from *I. ricinus* in Turkey (7), while the genetic difference between Th1 and *B. burgdorferi* (X15661) was much greater, 6.83%.

This report is the first of genetic detection of *B. valaisiana* in CSF, which indicates a probable association of this genospecies with disease in humans. *B. valaisiana* has been isolated from *I. ricinus* ticks collected from vegetation and from ticks engorged on birds, in several European countries, including Turkey (7). The pathogenic capabilities of *B. valaisiana* are still uncertain; it has been detected by PCR and restriction fragment length polymorphism analysis in skin biopsy specimens from two erythema migrans patients and from patients with mixed infection (erythema migrans and acrodermatitis chronica atrophicans) (4). Indirect evidence suggests that *B. valaisiana* is involved in some chronic clinical manifestations (8).

Borreliosis is difficult to diagnose by serologic evaluation and Western blot interpretation. In our patient, no intrathecal antibodies were produced to support clinical suspicion of disease. The low antibody titers could be attributed to antigenic variation between *B. valaisiana* and *B. burgdorferi sensu stricto*, which was used as antigen because no commercial kit is specific for *B. valaisiana*. Differences between the strain causing infection and the antigen may play a role in the false-negative results (9). The low antibody response in our patient could be caused by antimicrobial drugs and corticosteroid medication.

The high homology of the nucleotide sequence from our patient and respective *B. valaisiana* sequences from other European countries suggests that he likely was infected in Greece. The status of Lyme disease in southern Africa is unknown, but *Ixodes* spp. ticks have been found there, and preliminary evidence indicates that the disease may occur in humans in South Africa (10).

We detected *B. valaisiana* DNA in CSF of a patient with slow progressive spastic paraparesis, which suggests that this microorganism might be the causative agent of the disease. Nucleotide sequence information of *Borrelia* strains from clinical cases and ticks from different countries will elucidate the molecular epidemiology of the disease.

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Baylisascaris procyonis in California

To the Editor: We read with interest the article of Roussere et al. on the distribution of *Baylisascaris procyonis* eggs in northern California communities (1). The widespread dissemination and high density of raccoon latrines in residential areas clearly

pose potential health risks, particularly to young children.

While California has reported more cases of baylisascariasis than any other state, few published studies have reported on the distribution and prevalence of this helminth in the region. In 2001, we conducted a study to determine the presence of *B. procyonis* in the Santa Barbara area by examining roadkill raccoons recovered by animal control staff and stored in a refrigerated facility. On examination, the digestive tract from the stomach to the rectum was removed and tested for *B. procyonis* worms and eggs. Of 26 raccoons examined, 24 (92%, 95% confidence interval 75%–99%) were positive for *B. procyonis* infection. *B. procyonis* worms were found in 85% of the animals examined and eggs were found in 73%. Pet food was frequently found (43%) in the stomach contents of examined raccoons, indicating that such food was made accessible to these animals, either intentionally or inadvertently by residents.

B. procyonis has been identified along the central coast of California, which expands the known range of this helminthic zoonotic agent. This finding, coupled with other published studies, indicates that *Baylisascaris* may be prevalent throughout the state (1,2). Although our study was based on a small sample of selected raccoons, the high infection rate is cause for concern and indicates the potential for human exposure. A presumptive case of *B. procyonis* infection in an 11-month-old child was reported in Santa Barbara in 2003 (1).

Determining the distribution and prevalence of *B. procyonis* is necessary to inform local healthcare providers, public health authorities, and the public of the potential risk. Using road-kill raccoons is a relatively easy method for quickly assessing the presence of *B. procyonis* in a community. Also, this approach avoids trapping and handling live animals and allows stomach contents to be examined to determine where raccoons are feeding. Data from such assessments must be interpreted with caution, since they may not represent all raccoons in an area.

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Streptococcus iniae Discitis in Singapore

To the Editor: *Streptococcus iniae* is a well-recognized fish pathogen that can cause meningoencephalitis in tilapia and trout (1) and necrotizing myositis in red drum (2). We describe the first known human case of *S. iniae* infection in Singapore. This is the second report of spinal infection with this bacterium; however, commercial kits may misidentify *S. iniae*.

The first cases of *S. iniae* infection in humans were reported in Toronto, Canada, in 1995–1996 and included eight patients with bacteremic hand cellulitis and one patient with endocarditis, meningitis, and arthritis (3). Two additional cases were discovered retrospectively, a patient in Ottawa, Canada, with septic arthritis of the knee and a patient from Texas with bacteremic cellulitis. At least two more strains have been isolated from patients in Vancouver, Canada (4). Recently, Lau et al. described two cases of infection in Hong Kong. The first patient had bacteremic cellulitis; the second is recognized as the first patient with *S. iniae* osteomyelitis of the spine (5).

A 73-year-old female Chinese healthcare worker was admitted on October 5, 2003, to Singapore General Hospital. Her symptoms were fever for 3 days before admission and lower back pain that had progressively worsened for the past 2 months, causing her to become bedridden. She was ambulatory before the back pain started and had no history of a fall or injury to the back.

Upon examination, the patient's temperature was 37.1°C. She did not appear septicemic and was hemodynamically stable. No evidence of cellulitis was found, and neurologic examination of the upper limbs showed no abnormalities. Movement and strength of both lower limbs were limited by pain. Reflexes and plantar responses were normal, and no focal tenderness over the spine was found; chest x-ray results were normal. Laboratory tests showed the following: leukocytes 12.91 x 10^9/L, hemoglobin 9.9 g/dL, platelets 261 x 10^9/L, serum albumin 20 g/L, bilirubin 17 µmol/L, alkaline phosphatase 132 U/L, alanine transaminase 16 U/L, and aspartate transaminase 23 U/L. Renal function tests were within normal limits. The erythrocyte sedimentation rate was 115 mm/h, and C-reactive protein was 88.4 mg/L. No bacteria were grown from blood cultures. Treatment with empiric intravenous cefazolin was started.

Magnetic resonance imaging (Figure) of the patient's lumbar spine revealed discitis and osteomyelitis at the L3/L4 level, with associated epidural and paraverterbral abscesses. Anterior drainage of the abscesses and fusion with an iliac crest graft were performed on day 5 of hospitalization. Tissue samples obtained during this operation showed pus cells and gram-positive cocci. Cultures grew a β-hemolytic streptococcus which did not group with Lancefield groups A, B, C, D, F, or G antisera and was positive for pyrrolidonylarylamidase, negative for bile-esculin, and failed to grow in 6.5% NaCl. The API 20 Strep (bioMérieux, Marcy l'Etoile, France) identified the isolate as *S. dysgalactiae* subsp. *equisimilis* (profile number 4563117). Because *S. dysgalactiae* subsp. *equisimilis* are negative for pyrrolidonylarylamidase, we sequenced the 16S rRNA gene, which was identical to that of *S. iniae* ATCC29178 isolated in 1976 from a subcutaneous abscess of a captive Amazonian dolphin (6). The isolate was susceptible to penicillin, chloramphenicol, clindamycin, and vancomycin by antimicrobial disk diffusion tests (7).

The patient was asked specifically about exposure to fresh fish and other aquatic creatures before she became ill. She regularly prepared fresh fish bought from the local market and had sustained superficial cuts on her fingers before her back pain started. Though she did not recall any upper limb infection or previous septicemic episode, she likely had a transient bacteremia which spread to her lumbar spine.

The patient was given 2.4 g of intravenous penicillin every 4 hours for 6 weeks; she showed clinical improvement with decreasing erythrocyte sedimentation rate and C-reactive protein levels. At the time of discharge she was ambulatory with a walker.

Most confirmed cases of *S. iniae* infection to date have occurred in persons of Asian, predominantly Chinese, ethnicity. This phenomena is thought to result from a cultural preference for fresh, whole fish in cooking. Although *S. iniae* infection in fish is often associated with aquaculture, human infection has not been noted in fish-farm workers. A decline in immune function may be important in pathogenesis since most cases reported have been in elderly persons (mean age 70 years, range 40–81 years) (5).

The diagnosis of *S. iniae* infection may be missed because commercial identification kits do not include *S. iniae* in their databases. The Hong Kong and Singapore isolates were identified by the API 20 Strep system as *S. dysgalactiae* subs. *equisimilis*, and some Canadian isolates were identified by the VITEK system (bioMérieux, Marcy l'Etoile, France) as *S. uberis* (3). This problem may be compounded because physicians who are not familiar with the infection would not question the patient about exposure to fresh fish.

Apart from *S. iniae*, few other β-hemolytic streptococci are pyrrolidonylarylamidase positive. β-hemolytic enterococci and *S. pyogenes* are groupable with antisera to Lancefield group D and A antigens, respectively, and are easily identified by routine laboratory methods (8). *S. porcinus* is a rare human pathogen resistant to bacitracin, which produces a positive CAMP reaction and may react with antisera to Lancefield group B antigen (9).

All β-hemolytic streptococci that are ungroupable by Lancefield antisera should be tested for pyrrolidonylarylamidase. If this test is positive, the patient should be questioned about exposure to fresh fish, and identification of the isolate should be confirmed by molecular means.

A retrospective review of laboratory records showed that we had isolated β-hemolytic streptococci nongroupable with Lancefield groups A, B, C, D, F, or G antisera only five times in the last 2 years. Most of these were from respiratory sources and unlikely to be *S. iniae*. Therefore, we believe that *S. iniae* infection in Singapore is uncommon. However, the geographic range of this emerging zoonosis is likely to increase as clinicians and microbiologists become more aware of this pathogen.

The 16S rRNA gene sequence for this isolate has been submitted to GenBank (accession number AY581891).

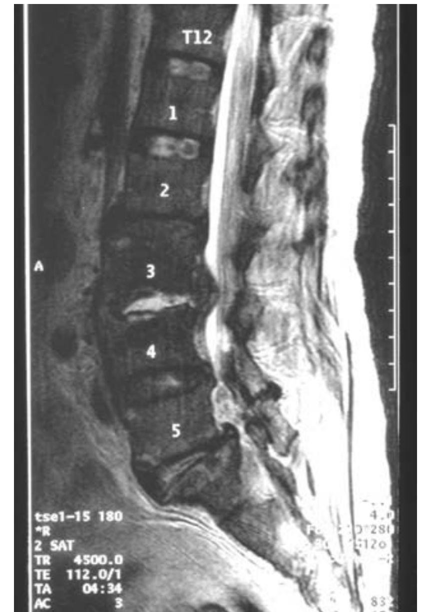

Figure. Magnetic resonance imaging scan of spine showing features compatible with discitis involving L3/L4 with surrounding paravertebral and psoas inflammation and epidural abscess.

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Rubella Epidemic Strain, Greece, 1999

To the Editor: A recent extensive study on global distribution of rubella virus genotypes by Zheng et al. (1) showed that most of the isolates tested were rubella genotype I (RGI) and that subgenotypes within RGI were apparent. Of these subgenotypes, three were currently active, one in the United States and Latin America, one in China represented by two specimens, and one international subgenotype that originated in Asia and spread to Europe and North America. More RGI subgenotypes, which have not yet been identified in specimen collections, may be currently active. In Zheng, et al. the distribution of rubella subgenotypes is shown; Greece is one of the four European countries where only RGI viruses were found. This letter provides more information about rubella in Greece and the strain that was responsible for the 1999 epidemic there.

Rubella virus is endemic in Greece. Vaccination in the private sector only was introduced in 1981 with a monovalent rubella vaccine. In 1989, a single dose of the measles-mumps-rubella (MMR) vaccine was introduced in the national vaccination program for 15-month-old infants. Because of the rubella outbreak of 1993, the vaccination policy changed. In 1997, a second vaccination was recommended for 11- to 12-year-old children. However, another major rubella epidemic occurred in 1999, beginning in late December 1998 and lasting until May 1999, with a peak in the number of cases in January. During this period, 1,438 rubella cases were reported throughout Greece; 765 were in the northern part of the country. In previous rubella epidemics in Greece, children were most affected. However, in the 1999 epidemic, a higher incidence rate was observed mostly among 15- to 19-year-old persons (2). During this epidemic, four cases of congenital rubella syndrome were reported. Because of this epidemic, the vaccination policy was revised. The new policy consists of two doses of the MMR vaccine, one to be given at 15 months of age and another to be given at 4–6 years of age (3).

During the 1999 epidemic, oral samples were collected from patients within a week of the onset of symptoms. Most of the samples were sent to Colindale, London, for testing; a few of them were stored at –70°C in our laboratory at the School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece. In 1999, four universities in the United Kingdom reported cases of rubella infection. Greek students were attending all of these universities; the students had spent the Christmas holidays in Greece and then returned to the United Kingdom. The U.K. rubella strains were identical to those of the Greek epidemic strain (4). We amplified and sequenced a 143-bp segment of the E1 gene by using reverse transcription-nested polymerase chain reaction from the samples stored in our laboratory (5). All 10 samples tested contained very similar or identical sequences, so we used one of them as the epidemic rubella strain. Comparing the Greek strain (Thess1/GRE99, accession no. AY540614) with rubella sequences taken from GenBank, we found that it belonged to the international (1997–2000) rubella RGI subgenotype. Although the genome region tested was short, the Greek rubella strain was highly homologous to the strains isolated from Germany in 1999 (G432/GER99, accession no. AF551761) and from Italy in 1997 (6423/PV/ITA97, accession no. AY161374). However, the Greek strain had a genetic difference of approximately 5% from strains isolated from Italy (4844/ITA93, accession no. AY161364), the United Kingdom (DNY/UNK93, accession no. AF039131) in 1993, and Germany (D075/GER92, accession no. AF039118) in 1992 and (G696/GER98, accession no. AY326342) in 1998. We also found that the Greek strain differed by 6% from the RA27/3 vaccine strain, predominantly used for RGI.

Zheng et al. (1) showed that the RGI-ITA97 genotype strain was

closely related to a genotypic group of viruses isolated from the eastern United States and two Caribbean countries from 1999 to 2001 (6). Results from our phylogenetic analysis showed that Thess1/GRE99, as well as strains G432/GER99 and 6423/PV/ITA97 (and the United Kingdom 1999 strains), were clustering with strains FRI/BAH97 (accession no. AY326359), isolated in 1997 from the Bahamas, and DES/MB-CAN97 (AY326358), isolated in 1997 from Manitoba, Canada (which also belong to the international 1997–2000 RGI genotype). These findings indicated that this rubella strain was circulating in both Europe and North America at least as early as 1997.

Recent data indicate that internationally circulating rubella viruses exist, even when vaccination programs are conducted. Comprehensive specimen collection and genotypic analysis are necessary to identify and track the emergence and spread of such genotypes.

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CTX-M β-Lactamase–producing *Escherichia coli* in Long-term Care Facilities, France

To the Editor: In long-term care facilities, most endemic infections affect respiratory and urinary tracts, as well as skin and soft tissues (1–3). Infection and colonization by antimicrobial-resistant organisms, in particular those producing plasmid-mediated extended-spectrum β-lactamases (ESBL), are common in long-term care facilities (4). Since 1984, ESBL-producing *Enterobacteriaceae* have spread among French hospitals; within Parisian public hospitals (Assistance Publique, Hôpitaux de Paris), ESBL-producing *Escherichia coli* is the most frequent species found, representing 49.5% of 220 *Enterobacteriaceae* isolated in 2002, mostly in urinary tract infections (5). Among ESBL-producing *Enterobacteriaceae*, CTX-M–type β-lactamases confer a higher level of resistance to cefotaxime and ceftriaxone than to ceftazidime. CTX-M–producing strains are endemic in Latin America, Japan, and certain parts of Eastern Europe; in contrast, these strains are emerging in France, Western Europe, and the United States (6). We report the first documented outbreak of CTX-M–producing *E. coli* infection in a long-term care facility in France. Our hospital is an 800-bed institution with 300 beds for long-term patients distributed among three units located in two buildings. The outbreak occurred in a 35-bed unit and involved 26 of 47 hospitalized patients from October 2001 to March 2003. This facility hosts patients for extended periods of time or permanently. The index case was identified in October 2001; the patient had a urinary tract infection attributable to an ESBL-producing *E. coli*, which showed resistance patterns not previously found in our hospital. Three new cases were detected within the following 2 months, and all patients had urinary tract infection with the same pattern of resistance. In January 2002, patients were screened for ESBL-producing strains by rectal swabbing and urine culture. The results showed *E. coli* with a high level of resistance to amoxicillin and ticarcillin (MIC > 128 µg/mL), partial restoration of susceptibility to these agents by addition of clavulanic acid (MIC = 16–32 µg/mL), and higher resistance to cefotaxime (MIC > 128 µg/mL) than to ceftazidime (MIC = 32–64 µg/mL.) A cephalosporin/co-amoxiclav synergy test was positive, which suggests a CTX-M ESBL. Strains were also resistant to ciprofloxacin (MIC 64 µg/mL) and gentamicin (MIC > 64 µg/mL) but remained susceptible to trimethoprim-sulfamethoxazole.

Attempts to transfer resistance to β-lactams by conjugation to *E. coli* J53-2 with the 26 strains tested were unsuccessful. In contrast, transformants were obtained with plasmid DNA of the 19 strains tested by electroporation. The transformants' susceptibility pattern was similar to that of the donor strains, except for ciprofloxacin resistance. Analytical isoelectric focusing showed that all

clinical strains and transformants had bands of β-lactamase activity with an alkaline pI of 7.6 and 5.4. Polymerase chain reaction (PCR) amplification of the 26 clinical isolates was positive for $bla_{CTX\text{-}M}$ and bla_{TEM} (7). The 26 strains of *E. coli* had the same profile by repetitive-element PCR and pulsed-field gel electrophoresis, while unrelated control strains had very different profiles. Sequencing in strains isolated from four of the patients identified a CTX-M-15 β-lactamase and a TEM-1 β-lactamase. The four strains were related to the phylogenetic group B2 and produced the iutA (ferric aerobactin receptor), YuA (*Yersinia* siderophore receptor), and fimH (type I fimbriae) virulence factors (8). Incidence of colonization or infection by the culprit strain was 34.3% (12 of 35 patients) within the initial 4-month period and 55.3% (26 of 47 patients) over a 1-year period.

Intensified hygienic procedures implemented in January 2002 contributed to a decrease in the number of cases in February only; since then, a regular increase of new cases extended the outbreak and caused problems with controlling it. All urinary tract infections were successfully treated with a 15-day course of trimethoprim-sulfamethoxazole; however, reinfection occurred in some. Neither incontinence ($p = 0.35$), dementia ($p = 0.22$), nor previous antibiotic treatment (amoxicillin, amoxicillin-clavulanic acid, extended-spectrum cephalosporins, and fluoroquinolones [p = 1.00, 0.30, 0.12, 0.52, respectively]) appeared to be risk factors for infection or colonization in our study, but the number of patients is too small to reach a conclusion. However, patients that were infected or colonized had greater functional impairment, especially incontinence and dementia. Nonambulatory status, decubitus ulcers, and feeding tubes were not risk factors for acquiring ESBL-producing *E. coli* in our study.

The outbreak has not been controlled: 13 patients have persistent digestive-tract colonization. Difficulties encountered in controlling such outbreaks may be explained by several factors. Patients cannot be easily isolated in long-term care facilities. Strict isolation and limitation of activity and mobility cannot always be applied because of their impact on social activities.

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Do Antiborrelial Antibodies Suggest Lyme Disease in Cuba?

To the Editor: Lyme disease is the most common vector-borne disease in the United States and parts of Eurasia (1). It represents a considerable emerging infectious disease threat because of its consequences to human health and the difficulties in preventing and controlling it (2,3).

In Cuba, Lyme borreliosis has never been reported. However, in the last 20 years ixodid ticks, mainly *Amblyomma cajennenses*, have been found in the human population in the Cuban village of Las Terrazas, Pinar del Río. These ixodid bites were frequent and widespread, especially in children, many of whom were hospitalized without a confirmatory laboratory diagnosis. Affected persons had symptoms associated with Lyme disease such as erythematous macules or papules, fever, fatigue, malaise, headache, arthralgias, myalgias, meningitis, peripheral radiculoneuropathies, and myocarditis (4).

A Cuban researcher, a specialist in

ixodid ticks, was bitten several times by the ticks; dermatologic and neurologic symptoms compatible with Lyme disease (skin lesions, hyperesthesia with loss of reflexes, loss of muscular coordination, and fecal incontinence) developed. Borreliosis was not diagnosed at this stage; the diagnosis was either myeloradiculitis or Guillain-Barré syndrome. Three years later, a serologic diagnosis of Lyme disease was made by indirect immunofluorescence in a laboratory in the Czech Republic (5).

During 1998, serum samples from 14 persons who lived in the village Las Terrazas and had epidemiologic and clinical evidence of Lyme disease, were studied in our laboratory. We used an immunoglobulin (Ig) G and IgM–enzyme-linked immunoabsorbent assay (ELISA) kit (Enzygnost Borreliosis, Behring, Marburg, Germany), in which each strip contained wells coated with inactivated borrelial antigen (detergent extract from strain isolate PKo [*Borrelia afzelii*]), to detect specific antibodies to *B. burgdorferi* complex. The assays were performed according to the manufacturer's instructions. In our study, five serum samples had positive IgM titers and one near the cutoff value by IgM and IgG.

ELISA has been widely used to detect antibodies to *B. burgdorferi*; however, this assay is not standardized, which results in different levels of sensitivity and specificity. False-positive results may occur, especially when serum samples are obtained from persons with other illnesses (6).

To study possible cross-reactions with other infectious illnesses, different serologic tests were applied to the positive serum samples by using ELISA. One sample was weakly reactive to human leptospirosis (indirect hemagglutination assay with erythrocyte-sensitive substance antigen [Labiofam, Havana, Cuba]), but no samples were reactive to syphilis (rapid plasma reagin [Imefa, Havana, Cuba] and hemagglutination of *Treponema pallidum* [Oxoid, Diagnostic Reagents, Basingstoke, UK]). No indication of other infectious diseases was found.

All serum samples positive by ELISA were also analyzed by IgG and IgM Western blotting in the spirochete laboratory at the University of Trieste, Italy. The Western blotting was performed with a protein profile from whole–cell strain PKo and by applying the criteria of positivity described by Hauser et al. (7). Two serum samples showed clear IgM antibody bands to 41- and 23-kDa proteins. No IgG bands were observed. This test reportedly is more sensitive than ELISA for IgM detection (6).

We investigated the clinical manifestations of the patients with positive Western blotting. We found that one of the patients had been bitten several times by ticks and had an erythematous rash around the different bite sites; the rashes reddened and expanded over the course of a few days, with partial central clearing. The patient also had fever, hepatosplenomegaly, adenopathies, joint pain, and some nonspecific symptoms. He was given erythromycin before the laboratory results were confirmed and had a satisfactory recovery. In similar situations, repeat testing would be highly advisable. This was the same patient with low levels of antibodies to *Leptospira*. Investigating the symptoms of the other patient was not possible.

The presence of IgM antibodies is frequently confirmed in the early stage of Lyme disease (6). The patient's history of being bitten by an *A. cajennenses* tick, clinical manifestations of Lyme borreliosis, and specific antibodies to *B. burgdorferi* complex suggest the diagnosis of Lyme disease.

A. cajennenses has not been reported as a vector for Lyme disease. However, it is very abundant and aggressive in Cuba, and bites from this species are common. The genus *Ixodes,* the main vector of *B. burgdorferi* sensu lato, has not been reported in the area of the study. Several articles describe a new species in the United States, *B. lonestari*. *B. lonestari* in *A. americanum* has been confirmed in humans with erythema migrans (8,9).

No serologic test is available for antibodies to *B. lonestari*. That we found antiborrelial-complex antibodies may suggest the presence of a new species in this antigenic complex containing cross-reactive antigens, but many other studies are necessary to confirm it. This study represents the first serologic report of antiborrelial antibodies in Cuba. It suggests that Lyme borreliosis is present and that new cases can be expected in our country. Further laboratory studies are necessary for a more accurate diagnosis of this emerging infectious disease in Cuba.

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Human Herpesvirus 6 Encephalomyelitis

To the Editor: Denes et al. (1) reports successful treatment of human herpesvirus 6 (HHV-6) encephalomyelitis. The patient was an immunocompetent young woman whose symptoms were fever, urinary retention, blurred vision, quadriparesis, bilateral papillitis, and optic neuritis. Magnetic resonance imaging (MRI) showed multiple lesions on the spinal cord white matter and the left thalamus, and the cerebral spinal fluid (CSF) showed inflammation. The patient was treated with acyclovir for 3 days, high-dose methylprednisolone for 5 days, cidofovir for 1 day, and ganciclovir for 15 days, starting on day 23 of hospitalization. By establishing a relationship between antiviral drug doses, serial determinations of HHV-6 DNA by polymerase chain reaction (PCR) in CSF, and neurologic improvement, Denes et al. concluded that antiherpesvirus drugs led to her recovery.

This case fits well in the spectrum of acute disseminated encephalomyelitis (ADEM), an inflammatory demyelinating disease of the central nervous systems of children and young adults, which occur in close temporal relationship with several infectious illnesses and immunizations (2–6). The disease has particular predilection to the optic nerves, spinal cord, brainstem, basal ganglia, and cerebral and cerebellar hemispheres. Maximal neurologic deficits are reached within several days, and resolution takes weeks or months. The condition is typically monophasic, but relapses have been reported (7). Histologic multifocal areas of inflammation and demyelinization are found. In the pathogenesis of ADEM, an initial injury caused by an infectious agent, followed by a secondary autoimmune response, has been postulated, and animal models have provided experimental support; both CD4 and CD8 T cells have been implicated in a secondary autoimmune response (6). Despite the lack of controlled studies, corticosteroids are widely used to treat ADEM and high-dose methylprednisolone is the drug of choice (3,4). The largest series of ADEM in adults included 40 patients with a mean follow-up period of 38 months. The patients were given a standardized treatment regimen of methylprednisolone, 500 mg daily intravenously for 5 days, with no additional therapy if they recovered completely. In patients with persistent neurologic deficits, the initial intravenous therapy was followed by a regimen of oral methylprednisolone, which was slowly tapered over 4 to 6 weeks. In patients with no response to this therapy, or whose condition had deteriorated during therapy, cyclophosphamide was given to seven patients, and immunoglobulin was given to one patient. Thirty-eight of 40 patients improved during the acute phase of the illness; in 7, symptoms completely resolved. One patient's condition remained unchanged and one patient died; no antiviral drugs were given (5).

The neurotropism of HHV-6 and that the CNS may be a site of viral persistence or latency are well recognized (8,9). On autopsy, evidence of fulminant encephalitis with HHV-6 DNA demonstrated by PCR, immunohistochemical staining, or nucleic acid hybridization, confirms that HHV-6 causes acute CNS disease (8). Nevertheless, whether evidence of HHV-6 DNA in CSF demonstrated by PCR can be solely relied on is debatable. HHV-6 DNA was detected in the CSF of 41 (28.9%) of 142 children with a history of HHV-6 infection (9). HHV6-DNA was detected in the CSF of 47 (61%) of 77 children examined after primary HHV-6 infection. In the remaining 30 children (39%), HHV-6 DNA was detected in both peripheral blood mononuclear cells and CSF samples. HHV-6 variant A was detected more frequently in CSF than in specimens of other sites, which suggests that HHV-6A has greater neurotropism (10).

The role of HHV-6 in acute multifocal neurologic disease in immunocompetent adults requires additional observation, and its role in multiple sclerosis is in question. Much can be learned from careful study of patients (1).

I caution the casual reader who may conclude that using antiviral drugs against herpes viruses is recommended when acute mutlifocal neurologic disease clinically compatible with ADEM is indicated. High-dose IV methylprednisolone is the most utilized treatment, and the patient in

the Denes et al. report was given it early in her hospital stay. The available evidence supports methylprednisolone as an essential drug in the management of ADEM.

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In Reply: The disease that we reported (1) was encephalomyelitis induced by a human herpesvirus 6 (HHV-6) reactivation. Our aim was to emphasize that HHV-6 can cause such a disease, even when the patient is immunocompetent, and to urge physicians to search for it.

Implicating HHV-6 in the pathogenesis of neurologic manifestations in the reported case can be challenged, as suggested by Dr. Soto-Hernandez (2). Polymerase chain reaction (PCR) results must be interpreted cautiously, especially in cases that lack corroborating clinical evidence of infection. In our case, the diagnosis was made initially when HHV-6 was found in the patient's cerebral spinal fluid (CSF) by using PCR, by the absence of other cause, and by our experience; adult CSF is usually negative for HHV-6 by using PCR. Moreover, in our case, clinical symptoms and HHV-6 in the patient's CSF evolved in the same way. The neurologic tropism of HHV-6 is well known, and the main manifestation in adults is encephalitis, especially in an immunosuppressed context. Diagnosis is usually based on finding HHV-6 genetic material in the CSF (3,4), which has now replaced brain biopsy. Positive tissue results do not distinguish latent from productive infections when PCR-positive CSF indicates viral particle production in the central nervous system (CNS). In our case, the absence of brain tissue did not allow immunohistochemical staining or in situ hybridization. In the study by Caserta et al. (5), cited by Dr. Soto-Hernandez, HHV-6 PCR was positive in CSF and negative in peripheral blood mononuclear cells in 28.9% of children $\leq$3 years old with prior HHV-6 infection. These results provide evidence of HHV-6 persistence in the CNS; this phenomenon is now well recognized. Nevertheless, HHV-6 persistence after primary infection is quite different from reactivation in an immunocompetent adult. High-avidity anti-HHV-6 immunoglobulin G detected in the patient's serum when the symptoms started proved that our patient had been infected with HHV-6 previously.

We appreciate Dr. Soto-Hernandez's suggestion concerning acute demyelinating encephalomyelitis (ADEM) in our case. ADEM is an inflammatory demyelinating disease of the CNS, occurring mostly in children and rarely in young adults, soon after an infection or a vaccination. The disease is often associated with exanthema. A virus is often thought to be the cause, but viral symptoms are often not documented and rarely treated. ADEM may evolve into multiple sclerosis, and HHV-6 has been proposed as one of the causes of that condition (6). For example, multiple sclerosis developed in 14% of children and 35% of adults with ADEM in the study by Schwarz et al. (7).

Spontaneous improvement of ADEM is regularly reported; however, when treatment is needed, especially during the acute phase, steroid therapy is frequently used. In our case, corticosteroids did not affect the evolution of the patient's neurologic symptoms. Conversely, introducing the antiherpes drugs (cidofovir and ganciclovir) was followed by improved clinical signs and negative results for HHV-6 in the CSF by PCR. Corticosteroids likely influence inflammation associated with ADEM, but if ADEM is the result of a viral infection with persistent viral replication, steroids might be deleterious, allowing an increase in viral replication (8,9).

In conclusion, we think that viruses, particularly HHV-6, should be considered in ADEM, even in immunocompetent patients. In case of a positive result, antiviral treatment must be given, eventually in association with corticosteroids. We cannot recommend using corticosteroids alone because of the risk of spreading the infection.

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Vancomycin Heteroresistance in Methicillin-resistant *Staphylococcus aureus*, Taiwan

To the Editor: In 1997, Hiramatsu and colleagues reported the first clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) showing reduced susceptibility to vancomycin (1). Soon thereafter, vancomycin-intermediate *S. aureus* (VISA) or heteroresistant VISA was reported to have disseminated in Japanese hospitals (2). In Taiwan, a survey of >5,000 clinical isolates of *S. aureus* at one tertiary medical center from 1999 to 2001 showed negative results for VISA or vancomycin-resistant *S. aureus* (VRSA) (3,4). We report the first two isolations of heteroresistant VISA in Taiwan.

In June, 2000, an 89-year-old man (patient A) with a history of cerebrovascular accident underwent ileal resection for ischemic bowel disease, and primary MRSA bacteremia developed during the hospitalization. Vancomycin was given for 14 days, and his fever rapidly abated. In October 2000, a Port-A-Cath (Smiths Industries Medical Systems, Deltec, Inc., St. Paul, MN) was inserted in the left subclavian vein. On June 14, 2001, he had another blood isolate of MRSA during an episode of *Enterococcus faecalis* bacteremia. Fever resolved after 4 days of intravenous vancomycin treatment (1 g every 12 h), and vancomycin treatment was continued for 21 days. Subsequent culture of blood drawn from the Port-A-Cath and peripheral veins on June 29 and July 6, 2001, did not yield any organism. MRSA bacteremia relapsed in November 2001, and the patient received intravenous teicoplanin treatment (400 mg every 2 days) for 3 weeks, and fever resolved rapidly. Transthoracic echocardiograpic tests showed no vegetation on the cardiac valves. The patient was hospitalized again in March 2002 because of relapsing MRSA bacteremia. The Port-A-Cath was removed, and culture of fluid from the indwelling pocket yielded MRSA. Fever and MRSA bacteremia persisted, with 17 sets of positive blood culture from March to May 2002, even under an adequate dose of intravenous vancomycin (serum trough level of vancomycin = 9–24 µg/mL and serum peak level = 18–30 µg/mL) and rifampin (600 mg/day). An infected thrombus over the subclavian vein was detected by venous duplex and thought to be an unresolved focus. Linezolid (600 mg every 12 h) was given for 10 days (April 30–May 9, 2002) but discontinued because of progressive thrombocytopenia. Vancomycin and rifampin were resumed on May 10, and positive blood culture with MRSA was noted on May 14. Oliguric renal failure developed in the patient on May 21 followed by shock, and he died on May 23.

A 72-year-old man (patient B) with coronary artery disease and chronic renal insufficiency underwent coronary artery bypass grafting and aortic grafting for abdominal aortic aneurysm in December 1999. The postoperative course was complicated with second-degree atrioventricular block and progressive renal failure. He was implanted with a permanent pacemaker and started long-term hemodialysis in March 2000. In April 2000, catheter (double lumen for hemodialysis)-related MRSA bacteremia developed in the patient. Vancomycin (1 g/week) was administered and the catheter was changed, but 17 successive episodes of MRSA bacteremia recurred from May to July 2000, despite an adequate serum level of vancomycin (trough level = 13–21 µg/mL and peak level = 24–38 µg/mL). Transesophageal echocardio-

graphy showed vegetation on the tricuspid valve. Vancomycin was changed to teicoplanin (400 mg every 3 days) because of vancomycin-associated skin rashes and eosinophilia, and the treatment was continued for 1 month. No MRSA infection was found in the subsequent 6 months. In January 2001, a subcutaneous abscess and osteomyelitis developed over the right humerus after the patient was injured in a fall, and bacteremia subsequently developed. Local debridement was performed, and glycopeptides were given for 6 weeks (vancomycin for 12 days and teicoplanin for 30 days). From March to April 2001, he had repeated episodes of MRSA bacteremia associated with pus discharge from the pacemaker insertion site. The pacemaker was removed, and high-dose teicoplanin (600 mg every 3 days) was given for 4 months, during which time MRSA bacteremia did not recur.

MICs of vancomycin were determined for the 21 isolates of MRSA from the two patients (Table) by the broth microdilution and agar dilution method using brain-heart infusion (BHI) agar or broth and Mueller-Hinton agar (MHA) or broth (BBL Microbiology Systems, Cockeysville, MD), according to the recommendations by the National Committee for Clinical Laboratory Standards (5). Vancomycin MICs were also determined by the Etest (AB Biodisk, Solna, Sweden) by swabbing 0.5 McFarland Standard on a BHI agar plate, and the results were read after incubation for 24 h. Mu3 and Mu50 were used as control strains. MICs of the following antimicrobial agents were also determined by using the agar dilution method: oxacillin (MHA plus 2% NaCl) and teicoplanin, fusidic acid, and linezolid (MHA).

All 21 isolates were highly resistant to oxacillin (MICs > 128 µg/mL) but susceptible to linezolid (MICs = 1–2 µg/mL) and fusidic acid (MICs = 0.06–0.25 µg/mL). Isolates with reduced susceptibility to vancomycin (MICs > 4 µg/mL, determined by more than one method) included A6 and A7 from patient A and B7 from patient B. Two isolates (B6 and B7) had Etest vancomycin MICs of 8 µg/mL, and one of them also had an MIC of 5 µg/mL by the agar dilution method (Table).

Analysis of the vancomycin-resistant subpopulation of two MRSA isolates (A7 and B7) from the two patients, one isolate (isolate C, Etest vancomycin MIC = 4 µg/mL) of MRSA recovered from a patient with bacteremia in 2000, and Mu3 was performed according to the description by Hiramatsu et al. (1,2). Heteroresistant VISA refers to isolates with vancomycin MICs for one or more subpopulations above the susceptible range (i.e., > 4 µg/mL) (1,2). Isolates

Table. Characteristics of 21 methicillin-resistant *Staphylococcus aureus* isolates recovered from two patients with recurrent bacteremia[a]

Designation of isolate	Date of isolation (mo/day/year)	Vancomycin MIC (µg/mL) Etest					Teicoplanin MIC (µg/mL) Agar dilution
		Etest	Broth microdilution		Agar dilution		Agar dilution
		BHI	BHI	MHB	BHI	MHA	MHA
Patient A (89-y-old man)							
A1	6/30/2000	3	2	1	2	2	2
A2	6/14/2001	4	3	1	2	2	2
A3	11/19/2001	4	4	2	2	2	2
A4	3/5/2002	4	3	1	2	2	4
A5	3/22/2002	4	3	2	3	2	4
A6	4/3/2002	6	5	4	4	2	4
A7	4/14/2002	6	5	4	4	3	4
A8	4/29/2002	5	4	2	4	3	8
A9	4/29/2002	6	3	2	4	3	4
A10	5/7/2002	6	4	2	4	2	4
Patient B (72-y-old man)							
B1	4/26/2000	3	2	1	2	1	2
B2	5/6/2000	4	2	1	2	2	8
B3	5/18/2000	6	4	2	4	3	8
B4	5/29/2000	5	4	1	4	2	8
B5	6/15/2000	6	3	2	4	3	8
B6	6/24/2000	8	4	2	4	3	8
B7	7/19/2000	8	4	3	5	3	8
B8	1/9/2001	4	1	1	2	1	1
B9	1/26/2001	4	2	1	2	1	1
B10	3/21/2001	3	2	1	2	1	1
B11	4/6/2001	4	2	1	2	1	1
Mu3		3	3	2	3	2	–
Mu50		10	8	5	8	4	–

[a]BHI, brain heart infusion; MHB, Mueller-Hinton broth; MHA, Mueller-Hinton agar.

of A7 and B7, like the Mu3 strain, contained resistant subpopulations that grew in >4 μg/mL vancomycin and were thus considered as heteroresistant VISA strains (Figure).

Pulsed-field gel electrophoresis analysis after digestion of chromosomal DNA with *Xba*I showed that 10 isolates from patient A belonged to pulsotype a and those from patient B belonged to pulsotype b (Table). Heteroresistant VISA isolates were genetically indistinguishable from vancomycin-susceptible isolates.

This report is the first of heteroresistant VISA causing clinical infection in Taiwan, although the clinical importance of heteroresistant VRSA infection is unclear. While a previous report described no treatment failure of patients infected with heteroresistant VRSA strains (6), another study found higher death rate in patients infected with vancomycin-heteroresistant staphylococci (7). A recent case-control study of varying degrees of vancomycin susceptibility in MRSA bacteremia did not conclude whether a clinical difference was noted between bacteremia attributable to heteroresistant VISA and homogeneously susceptible strains (8). This report describes recurrent bacteremia caused by a single clone of MRSA that possessed subpopulations with different glycopeptide susceptibilities during different periods of treatment. These heteroresistant VISA strains were associated with prolonged glycopeptide use and glycopeptide treatment failure. Biofilm formation in the implanted intravascular devices may explain the relapsing nature in these two patients (9), and these heteroresistant VRSA strains might contribute to lack of bacteriologic eradication in infected valves and intravascular thrombi (10). Linezolid, having good in vitro activity against MRSA with reduced susceptibility to vancomycin, still failed to eradicate the organism within the infected thrombus in patient A.

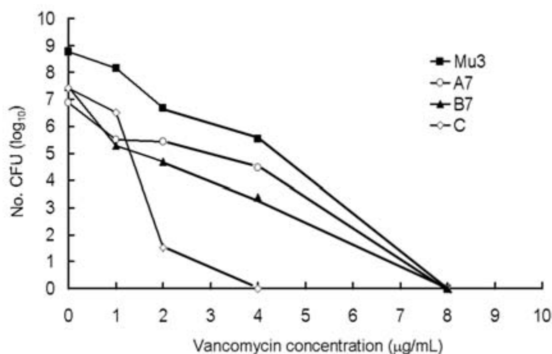

Figure. Population analysis of Mu3, two methicillin *Staphylococcus aureus* (MRSA) isolates (isolates A7 and B7) with heteroresistance to vancomycin, and one vancomycin-susceptible MRSA (isolate C).

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A Clinician's Dictionary of Pathogenic Microorganisms

James H. Jorgensen and Michael A. Pfeller, authors

ASM Press, Washington, DC
ISBN: 1-55581-280-5
Pages: 273, Price: US $29.95

This dictionary of pathogenic microorganisms, published by the American Society for Microbiology, is simple and useful. This book is divided in four sections, bacteria, fungi, parasites, and viruses. Each organism is presented alphabetically in its section. Older names are mentioned and connected with current names. A brief bibliography is also provided at the end of each chapter.

The emergence of new infectious agents in the last 2 decades makes it difficult for clinicians to recognize new diseases and new names. A memorandum to address this matter would have been useful. Moreover, the genomic revolution has caused a taxonomic revolution; this is specifically true for bacteriology. For example, 16S rRNA sequencing allowed reclassification of many pathogenic organisms and descriptions of many others. These advances is genomic knowledge have brought about many changes in the names of pathogenic microorganisms, evidenced here by the authors devoting the largest part of the book to bacteria.

The information provided, although very brief, is usually complete enough to provide a basic understanding of the microorganism. Many new organisms such as *Ehrlichia* and monkeypox viruses, as well as emerging diseases such as severe acute respiratory syndrome, are included.

This book provides basic information clinicians need for a quick reference book. It largely succeeds in this attempt and may be very useful as a pocket book for nonspecialists at the patient's bedside. I recommend it for general practitioners and health professionals.

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Cryptosporidium: from Molecules to Disease

R.C. Andrew Thomson, Anthony Armson, and Una M. Ryan, editors

Elsevier, Amsterdam
ISBN: 444-51351-5
Pages: 422, Price US $139.00

The protozoan parasite, *Cryptosporidium*, has recently emerged as a human pathogen. It was unidentified or unrecognized as a cause of illness in humans until 1976. Since then, it has caused gastrointestinal illness around the world. Its small size, low infectious dose, resistance to chlorination, and durability in the environment has made it a uniquely challenging organism for environmental scientists and public health professionals.

This book includes full text of abstracts and invited papers from an international conference held in Australia in October 2001. More than 100 scientists from more than 15 countries contributed to the conference.

The "from molecules" aspect of the book, which addresses molecular and

biochemical features of the life cycle, infection, and detection of *Cryptosporidium*, gives a complete picture with detailed papers and abstracts of subjects, including pathogenesis and immune response, cell culture methods, detection methods, and molecular taxonomy. The main focus of the book is on descriptions and evaluations of traditional and novel methods to detect and differentiate *Cryptosporidium*. Papers are also included that describe methods of detecting *Cryptosporidium* in environmental water samples, detail surveys that determine the occurrence of *Cryptosporidium* in water supplies, and explain how to acquire laboratory accreditation for testing water samples.

The book focuses less on understanding the public health aspects of *Cryptosporidium*, its epidemiology, and treatment for the illness it causes. Notably absent are descriptions of serologic assays used for detecting *Cryptosporidium* in surveillance and epidemiologic studies. Recent studies have identified a high seroprevalence in the general population, which indicates that infection may be widespread (1–5). Including examples of quantitative microbial risk assessments would have been useful (6). These assessments are logical extensions of the valuable human infectivity studies described in several papers in the book. The treatment portion presents interesting results of randomized trials of nitroaxanide therapy but is otherwise limited.

The organization and grouping of the papers and abstracts were confusing. An introduction and summary for each section to help the reader identify and assimilate the information in an organized manner would have been helpful.

Despite these shortcomings, this book assembles and summarizes an impressive array of recent advances in *Cryptosporidium* research. I recommend this book for laboratory scientists, microbiologists, laboratory technicians, and water-quality professionals. Medical professionals involved with research to detect and differentiate *Cryptosporidium* will likely find this book useful. Because of the technical nature of the papers and the emphasis on microbiologic methods, the book will be less useful for public health professionals, risk managers, and epidemiologists. Because of the rapid progress of *Cryptosporidium* research, I recommend using this book as one reference but also conducting a broad search of current literature for new studies or additional advances.

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Conference Summary

Transnational Issues in Quarantine

How to promote transnational collaboration in implementing quarantine was the topic of a January 19–23, 2004, conference sponsored by the Defense Threat Reduction Agency. Fifty invited participants discussed the status of quarantine planning in 13 countries (the Americas, Israel, and several members of the European Union [EU] nations). Held in the Wilton Park Conference Centre, Sussex, United Kingdom, the conference, "Quarantine following an International Biological Weapons Attack: Building Cooperation, Achieving Consistency," also addressed quarantine in response to emerging infectious diseases.

Participants first examined the legal foundation for quarantine in their countries. Federal Canadian quarantine law applies only to national ports of entry or exit; provincial laws govern quarantine in the provinces. The U.S. Centers for Disease Control and Prevention has quarantine responsibilities at national ports of entry or departure; this agency may also become involved when a disease is spreading across state borders or even within a state (when invited by the governor of the state or ordered by the U.S. Secretary of Health and Human Services or the U.S. President). In general, however, quarantine in the United States is a local or state government issue. Quarantine laws in these jurisdictions vary, and some public health authorities expressed reluctance to address their shortcomings through legislation for fear that skeptics of quarantine would further weaken the laws. Other nations would turn to the World Health Organization and its International Health Regulations of 1969 (IHR) for guidance. A revised IHR should be available by 2005, but currently it lists only three diseases—plague, cholera, and yellow fever—as subject to quarantine and offers scant help in planning quarantine. Thus, the legal framework for quarantine varies and contributes little to the construction of a consistent approach to quarantine among nations.

European public health officials have forged some bilateral cooperative agreements and are discussing establishing a regional disease control center for EU nations. They are not, however, developing and testing national or transnational plans for possible large-scale quarantine. Some participants thought that consistency in developing and implementing quarantine measures was not necessarily desirable, given that each nation must deal with threats in accordance with its own culture, laws, and traditions. Others thought that inconsistencies in response to the same disease threat might encourage persons to question the need for quarantine measures and choose not to comply. The United States also has not developed comprehensive quarantine plans, trained staff, or conducted quarantine exercises in local communities, despite recently issued federal quarantine guidelines. Especially lacking are processes and procedures to clarify decision-making and coordination in communities with multiple jurisdictions.

The heightened concern of the United States about bioterrorism was not shared by others at the conference, although all agreed that persons would likely demand a federal response to a health crisis caused by terrorists, including any required quarantine. Other issues discussed included assurances of compensation for income lost while in quarantine (strongly recommended as a component of any quarantine plan) and psychosocial support to reduce the sense of isolation experienced by many persons while in quarantine. Officials with information management experience during health- and nonhealth-related crises commented on the need for caution in making public statements when faced with a new and evolving threat.

The conference permitted participants to establish working relationships with one another, but it also highlighted gaps in comprehensive transnational quarantine planning. The complete conference report is available at http://www.dtra.mil/about/ASCO/wpc/wpc.cfm

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Conference Summary

Ethics and Epidemics

More than 90 people attended a March 25–27, 2004, conference on Ethics and Epidemics. This conference was sponsored by the Albany Medical College–Graduate College of Union University Masters in Bioethics Program, the University at Albany School of Public Health, the New York State Department of Health, and the Wadsworth Laboratories. Attendees came from Australia, Africa, Asia, Europe,

Canada, and the United States. Among the 24 papers and panels, presentations were made by George Annas, professor and chair of Health Law at the Boston University School of Public Health; Ezekiel Emanuel, chair of the Department of Clinical Bioethics of the Magnuson Center of the National Institutes of Health; Thomas R. Freiden, commissioner, New York City Department of Health; Matthew Wynia, director of the Institute of Ethics of the American Medical Association; Kenyan bioethicist Angela Wassuna, associate for International Affairs of the Hastings Center; and 19 other bioethicists and health professionals.

Presentations ranged from case studies to health policy debates. Many reviewed the history of epidemics, emphasizing their global nature and the imperative of global strategies for epidemic control. Several papers examined recent epidemics and explored new strategies for dealing with epidemic control while respecting human rights. The consensus was that the old policeman model of public health needs updating. Discussion focused on how best to balance public safety, professional responsibility, personal liberty, and human rights, while effectively containing epidemics. Emanuel and Wynia reaffirmed the responsibility of physicians and first responders to put their health and lives at risk in combating epidemics. Yet, noting the vulnerability of first responders (in the Toronto severe acute respiratory syndrome [SARS] outbreak and elsewhere), they distinguished between bravery and foolhardiness, arguing that just as professionals have a responsibility to protect the public from disease, the public, in turn, has a responsibility to provide the training, equipment, and resources to minimize the need to take risks.

Virtually all conferees observed that the public health infrastructure needs substantial rebuilding to cope effectively with epidemics. Annas, however, noted that in bioterrorist assaults, the control of biologic agents is a public health problem to be dealt with by public health officials, not by the U.S. Department of Defense or the U.S. Department of Homeland Security. He further stated that policies on epidemic control that involve consistent, open, and truthful communication with the public—like those used in New York and Toronto during the recent SARS outbreak—create cooperative environments that minimize conflicts between freedom and safety and limit the effects of isolation and quarantine. However, Emanuel et al. asserted that the traditional enforcement authority of public health law was essential and needed as a fallback. The result of the debate was that 21st century methods need to be developed to control infectious disease epidemics that reconcile the need to protect public health and respect human rights.

The conference program is available on http://www.bioethics.union.edu under "News." For further information contact bioethics@union.edu or 518-388-8045.

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Correction, Vol. 10, No. 7

Correction heading for "Murine Typhus with Renal Involvement in Canary Islands, Spain" by Michele Hernandez-Cabrera et al. was inaccurate. Article appeared in Vol. 10, No. 4.

Correction heading for "Bovine Spongiform Encephalopathy Infectivity in Greater Kudu (*Tragelaphus strepsiceros*)" by Andrew A. Cunningham et al. was inaccurate. Article appeared in Vol. 10, No. 6.

We regret any confusion these errors may have caused.

Correction, Vol. 10, No. 8

For the article by Michael Aquino et al., p. 1499, the correct title is "Protective Behavior Survey, West Nile Virus, British Columbia."

The corrected article appears online at http://www.cdc.gov/ncidod/eid/ vol10no8/03-1053.htm

We regret any confusion this error may have caused.

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Measurable Indicators and Public Health

Polyxeni Potter

At its deepest level, reality is mathematical.
–Pythagoras

Art came easily to Hans Holbein the Younger. Son and student of master painter Hans Holbein the Elder, he showed extraordinary talent at a young age in his native Augsburg, a bustling commercial town in southern Germany. Fascination with Italian renaissance took him to Lombardy, where he studied the work of Leonardo da Vinci and the portraits of Lorenzo Lotto. While still a teen, Holbein moved to Switzerland and became established in Basel, where he met Erasmus of Rotterdam and other humanists under whose generous patronage he began an illustrious career as portrait and religious painter (1).

"The arts here are freezing," wrote Erasmus to Thomas More and other friends in England, urging them to support Holbein when he moved to London from Switzerland amidst the turmoil of the Reformation (2). Events of the day, among them Britain's break with the Catholic Church and the dissolution of monasteries, altered the art scene. While patronage and demand for images of religious content declined, Holbein flourished as court painter for King Henry VIII, producing woodcuts, glass and other decorative designs, and timeless portraits of the intellectual aristocracy, until his death of the plague in 1543 (3).

Part of the brief but brilliant movement known as renaissance of the North, which included Albert Dürer and Mathias Grünewald, Holbein was able to grasp and depict the human image in a way that eluded his contemporaries (4). His portrait of Erasmus captured the essence of the famous author whose uncharismatic physique had frustrated other artists. And his single surviving portrait of Henry VIII created an enduring perception of the notorious monarch for posterity.

Holbein's celebrated portraits recorded more than the physical appearance of luminaries of his age. Many, including the portrait of Nicholas Kratzer, on this month's cover of Emerging Infectious Diseases, place the sitter in a topical context, providing valuable character clues and social commentary. Kratzer, mathematician and astronomer to Henry VIII and friend to Holbein, was a prominent maker of sundials and clocks (5). These popular objects represented practical application of mathematics and symbolized scientific knowledge, a notion wildly appreciated long before it was fully understood.

In Holbein's painting, Kratzer is preoccupied. His trancelike expression reflects detachment and lapse into some unknown calculation, an abstract reality whose nature is alluded to by the instruments at the periphery of the portrait. His smooth hands seem skilled and confident around the elaborately drawn geometric figures and the mathematical tools strewn provocatively in the foreground.

Holbein was a deliberate observer. He sorted the evidence of

physical reality that he so fastidiously gathered for internal character clues. In his portraits, the stubble on the chin or smudge on the thumb was intentional, and the painstaking collection of minute and precise detail built a composite larger than its parts. This intricate composite, much often missed by the casual eye, was purposeful and focused. Free of extraneous or distracting elements, it dispassionately laid out for the viewer a meticulous image to probe for inner meaning and final interpretation. Selectively descriptive, proportional, fully cognizant of order and balance, his portraits offered a glimpse into a person's soul and an unadulterated version of the artist's perception of reality.

Domain of the artist, observation is equally domain of the scientist. Fueled by the desire to know, it drives systematic collection of data, the facts needed to formulate a unified concept of nature and the laws that govern it (6). Scientific observation, like Holbein's artistic equivalent, goes beyond the chaotic collection of facts. Sufficiently ascertained and methodically arranged and analyzed, facts form mathematical models, create measurable indicators, predict impact, and calculate costs to produce meaningful and applicable public health models. When graced with clarity of expression, like Holbein's portraits of distinguished humanists or John Snow's geospacial maps of cholera cases, observation produces good art and good science.

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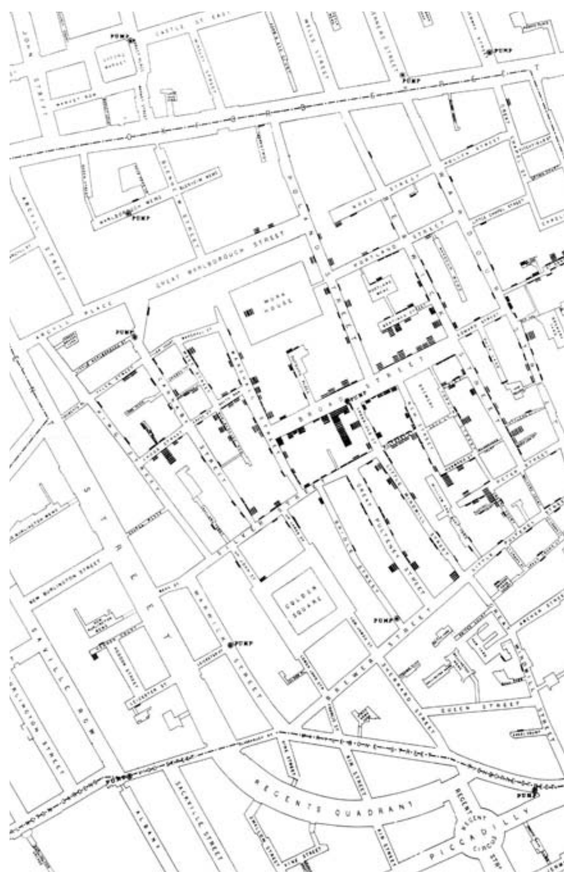
Editor's note: September 2004 marks the 150th anniversary of Dr. John Snow's meticulous studies of cholera in London, which culminated in the iconic removal of the handle from the Broad Street pump.

"The deaths which occurred during this fatal outbreak of cholera are indicated in the accompanying map, as far as I could ascertain them. There are necessarily some deficiencies…. The deficiencies I have mentioned, however, probably do not detract from the correctness of the map as a diagram of the topography of the outbreak; for, they would probably be distributed over the district of the outbreak in the same proportion as the large number which are known…. The pump in Broad Street is indicated on the map, as well as all the surrounding pumps to which the public had access at the time. It requires to be stated that the water of the pump in Marlborough Street, at the end of Carnaby Street, was so impure that many people avoided using it. And I found that the persons who died near this pump in the beginning of September, had water from the Broad Street pump. With regard to the pump in Rupert Street, it will be noticed that some streets which are near to it on the map, are in fact a good way removed, on account of the circuitous road to it. These circumstances being taken into account, it will be observed that the deaths either very much diminished, or ceased altogether, at every point where it becomes decidedly nearer to send to another pump than to the one in Broad Street."

From On the Mode of Communication of Cholera, 1854[1]

[1]Snow J. Snow on cholera. New York: Hafner Publishing Company. 1965.



Map of the Broad Street pump cholera outbreak of 1854 courtesy of the University of California, Los Angeles, School of Public Health, Department of Epidemiology. For more information, see http://www.ph.ucla.edu/epi/snow.html

EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends
Vol. 10, No. 10, October 2004

Upcoming Issue

Look in the October issue for the following topics:

Current Epidemiology of *Pneumocystis* Pneumonia

Dihydropteroate Synthase Gene Mutations in *Pneumocystis* and Sulfa Resistance

Strain Typing Methods and Molecular Epidemiology of *Pneumocystis* Pneumonia

West Nile Virus Economic Impact, Louisiana, 2002

Virus-specific RNA and Antibody from Convalescent SARS Patients Discharged from Hospital

Fluoroquinolone Resistance in Penicillin-resistant *Streptococcus pneumoniae* Clones, Spain

Sulfa Use, Dihydropteroate Synthase Mutations, and *Pneumocystis* Pneumonia

Dengue Emergence and Adaptation to Peridomestic Mosquitoes

Escherichia coli and Community-acquired Gastroenteritis, Melbourne, Australia

Susceptibility to ST11 Complex Meningococci Bearing Serogroup C or W135 Polysaccharide Capsules, North America

Genetic and Transmission Analysis of *Helicobacter pylori* Strains

Complete list of articles in the October issue at http://www.cdc.gov/ncidod/eid/upcoming.htm

Upcoming Infectious Disease Activities

September 13–16, 2004
ICNA International Conference and Exhibition 2004
Belfast, Northern Ireland
Contact: 0161-301-6857 or icna@comtec-presentations.com
http://www.icna.co.uk

September 19–23, 2004
Extremophiles 2004
American Society for Microbiology
Cambridge, MD
http://www.asm.org/Meetings/index.asp?bid=19177

September 23–26, 2004
JPGM Gold Con
Parel, Mumbai, India
Contact: Atul Goel, Editor, Journal of Postgraduate Medicine, 91-22-24129884 or goldcon@jpgmonline.com
http://www.jpgmonline.com/goldcon.asp

October 27, 2004
Public Health Research Institute Symposium - Infectious Diseases: Global Threats to Public Health
Newark, NJ, USA
http://www.phri.org/news/news_events50526.asp

October 30–November 2, 2004
44th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)
Washington, DC, USA
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http://www.icaac.org

November 7–11, 2004
53rd Annual Meeting, American Society of Tropical Medicine and Hygiene
Miami, FL, USA
Contact: 847-480-9592 or astmh@astmh.org

EMERGING INFECTIOUS DISEASES

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West Nile Virus

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EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit http://www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://www.cdc.gov/eid/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/ EID/style_guide.htm).

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. 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 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but 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 one 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 (effective January 2005). 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.