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**On the Cover:** Eugene Delacroix (1793-1863). Arab horses fighting in a stable (detail). 1860. Oil on canvas. Photo: Gerard Blot.



Louvre, Paris, France

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# Use of Automated Ambulatory-Care Encounter Records for Detection of Acute Illness Clusters, Including Potential Bioterrorism Events

**Ross Lazarus,*† Ken Kleinman,‡§ Inna Dashevsky,‡ Courtney Adams,‡ Patricia Kludt,¶ Alfred DeMaria, Jr.,¶ and Richard Platt*‡§**

The advent of domestic bioterrorism has emphasized the need for enhanced detection of clusters of acute illness. We describe a monitoring system operational in eastern Massachusetts, based on diagnoses obtained from electronic records of ambulatory-care encounters. Within 24 hours, ambulatory and telephone encounters recording patients with diagnoses of interest are identified and merged into major syndrome groups. Counts of new episodes of illness, rates calculated from health insurance records, and estimates of the probability of observing at least this number of new episodes are reported for syndrome surveillance. Census tracts with unusually large counts are identified by comparing observed with expected syndrome frequencies. During 1996–1999, weekly counts of new cases of lower respiratory syndrome were highly correlated with weekly hospital admissions. This system complements emergency room- and hospital-based surveillance by adding the capacity to rapidly identify clusters of illness, including potential bioterrorism events.

Rapid identification of unusual clusters of acute illness in the general population is a fundamental challenge for public health surveillance (1). Recent distribution of *Bacillus anthracis* spores and the resulting occurrence of clinical disease (2) provide new impetus to developing and implementing surveillance systems that can identify both bioterrorism events and naturally occurring illness clusters, such as influenza and waterborne disease. Recognizing individual cases of infection, e.g., inhalational anthrax, requires astute and alert clinicians. However, many potential biological agents of terrorism, including anthrax, have nonspecific prodromal phases, and no explicit diagnosis is ever made for many other syndromes of potential importance. Recognizing these clusters at the earliest possible opportunity will require well-designed surveillance systems to ensure timely detection of unusual clusters of prodromal, nonspecific illness.

Several projects have been developed specifically to provide improved surveillance for detecting bioterrorism in urban populations (3). Some of these existing surveillance systems operate in emergency departments and hospitals (4). While these systems are very useful, implementation may be impeded by the effort required for timely collection and analysis of diagnosis data in a suitable format. Additionally, emergency rooms and hospitals may see increased numbers of cases days after the first, milder symptoms of disease bring new patients to ambulatory-care settings.

Surveillance systems based in ambulatory-care settings, particularly those based on automated medical records, may therefore provide worthwhile additional information. One of the best-known such systems is the Department of Defense Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE) system (5), which is based on encounter data from health services operated by the Department of Defense. Another such system is operating in Minnesota (6). Nurse hot lines have also been used for surveillance purposes (7).

We describe here an automated system developed in a partnership between the Centers for Disease Control and Prevention, the Massachusetts Department of Public Health, a large group practice, a health plan, and an academic department. The system produces next-day information about illness clusters, based on ambulatory-care visits and telephone calls.

## Methods

The utility of diagnoses from automated ambulatory encounter data for detecting respiratory disease clusters has been described (8). In this report, we extend the use of encounter data to produce daily surveillance summary reports covering a broad range of syndromes for use by public health officials and health-care providers.

The encounter data come from an electronic medical record system used by Harvard Vanguard Medical Associates,

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a large multispecialty group practice, to record all ambulatory-care encounters, including telephone contacts, regular visits, and urgent-care encounters, but not emergency room visits. The practice serves approximately 250,000 members, representing approximately 10% of the population of eastern Massachusetts.

The automated record system is a commercial product (Epicare; Epic Systems Corporation, Madison, Wisconsin; available from: URL: http://www.epicsys.com) used by many large medical groups. It represents a valuable source of surveillance data because it operates in real time (i.e., records are updated as information is entered). Additionally, to the extent that practices engage in some form of prepaid care, the population served can be explicitly enumerated; the surveillance report described below is restricted to approximately 175,000 members of Harvard Pilgrim Health Care, a principal health maintenance organization in the region. These persons constitute a defined population that receives essentially all its ambulatory care in this practice. Demographic information and addresses are available for all these persons. At the time of consultation, clinical diagnoses are assigned for each encounter by the clinician, who chooses from lists of terms on the encounter screen; essentially all episodes are coded by the end of the same day on which care is given. Although an unlimited number of codes can be chosen, approximately 90% of encounters have three or fewer codes assigned (8), stored as ICD-9 codes. Each night, an extract is created of all encounters recorded in the previous 24 hours with any of >1,500 ICD-9 codes in any of the syndrome categories. The patient's temperature is also recorded along with the ICD-9 codes. Demographic data are merged with each record through a link to the patient's membership record.

As a way of grouping insured persons into neighborhoods, the addresses of the insured plan members, obtained from the HMO's data, have been coded by Geographic Information System (Mapping Analytics, Rochester, NY) to determine the census tracts of their residences (9,10).

### Developing and Defining Syndromes

Patient encounters are categorized into syndrome groups according to the ICD-9 codes assigned at the time of consultation. The surveillance software considers each encounter record in turn and merges related ICD-9 diagnosis codes into syndrome groups by using a modification of a provisional classification scheme developed as part of the ESSENCE project (5). This scheme reduces the complexity of the ICD-9 into eight syndrome categories: coma/shock, neurologic, upper gastrointestinal, lower gastrointestinal, upper respiratory, lower respiratory, dermatologic, and sepsis/fever. We made two major modifications of the syndrome definitions: the number of ambulatory episodes in the coma/shock category was almost zero in 4 years of data we examined, so it was combined with the neurologic syndrome category. A new syndromic category representing diagnoses of Centers for Disease Control and Prevention (CDC) bioterrorism category A agents (11) (anthrax, botulism, plague, smallpox, tularemia, and hemorrhagic fever) is reported separately. We also added an additional influenza-like illness category, defined by the CDC sentinel surveillance definition of fever >37.8$^{o}$C measured in the office plus cough and/or sore throat in the absence of a known cause (12).

An individual patient may have multiple encounters associated with a single episode of illness (e.g., initial consultation, consultation 1–2 days later for laboratory results, and follow-up consultation a few weeks later) (8). To avoid double counting from this common pattern of ambulatory care, the first encounter for each patient within any single syndrome group is reported, but subsequent encounters with the same syndrome are not reported as new episodes until $\geq$6 weeks has elapsed since the most recent encounter in the same syndrome. We have reported that grouping respiratory illness visits into episodes reduces the total number of events by 38% in this clinical setting (8). This practice of grouping clinical encounters into episodes of illness occurs independently for different syndromes. For example, a patient could qualify for two different syndromes on a single visit if codes for cough (lower respiratory syndrome) and diarrhea (lower gastrointestinal syndrome) are assigned at the same visit; or a lower gastrointestinal syndrome episode could begin a few days after the start of a lower respiratory syndrome episode.

### Reporting Results

A daily surveillance summary report (Tables 1 and 2; Figure 1) was designed in collaboration with staff from the Massachusetts Department of Public Health and the medical group's administration, which has operated since it was implemented on October 25, 2001. The aim of the report was to identify any unusually large numbers of episodes of illness within the ambulatory-care system. The current version (Table 1) shows new episode counts and rates (per 1,000 insured persons) for all syndromes combined and for each individual syndrome, during the previous day. Mean rates are presented for the same day of the week in the same month of the previous 2 years, as well as the statistical probability associated with these counts derived from a generalized linear mixed model (described in the Models and Analysis section) for the four most common syndromes.

Each day's report also includes a list and maps of the residence locations of cases with respiratory and gastrointestinal syndromes (Tables 1 and 2; Figure 1). The list and the map both show the five census tracts in the region with the most improbably large number of new episodes, based on the statistical model described. Daily updates are disseminated to authorized persons through a password-protected area on a Secure Sockets Layer (SSL) (13,14) encrypted website.

### Models and Analysis

For each syndrome, we used a generalized linear mixed model (GLMM) (15–17) to model the daily counts from local neighborhoods over a 4-year historical period. In our model,

Table 1. Daily public health surveillance report of office visits with diagnoses corresponding to infection syndromes: summary report for Monday, March 4, 2002, Massachusetts

| Syndrome | Rate/1,000 health plan members (no. of visits)[a] | Probability[b] | 1999 average rates for this weekday in the same month | 2000 average rates for this weekday in the same month |
|---|---|---|---|---|
| All combined | 2.015 (328) | | 1.918 | 2.123 |
| Upper respiratory | 1.087 (177) | 0.999 | 1.151 | 1.251 |
| Lower respiratory | 0.405 (66) | 0.999 | 0.369 | 0.474 |
| Upper gastrointestinal | 0.166 (27) | 0.999 | 0.094 | 0.110 |
| Lower gastrointestinal | 0.227 (37) | 0.999 | 0.221 | 0.173 |
| CNS/neurologic[c] | 0.000 (0) | | 0.003 | 0.007 |
| Dermatologic | 0.012 (2) | | 0.023 | 0.022 |
| Sepsis/fever | 0.000 (0) | | 0.057 | 0.086 |
| Influenza-like illness | 0.117 (19) | | — | — |
| CDC bioterrorism category A agents[d] | 0.000 (0) | | 0 | 0 |

[a] Excludes individuals' repeated visits within 6 weeks for the same syndrome.
[b]Probability of at least this many episodes occurring at least once per year, when the data are adjusted for month, day of week, holidays, secular trend, and variability among census tracts
[c]CNS, central nervous system; CDC, Centers for Disease Control and Prevention.
[d]Anthrax, botulism, plague, smallpox, tularemia, and hemorrhagic fever.

census tracts (CT) form the neighborhoods, but this unit can be extended easily to larger or smaller geographic units if desired. Sample SAS code is provided in Appendix 1. The model closely resembles logistic regression, so that the logit, $\log(\Pr(y_i = 1) / \Pr(y_i = 0))$, is modeled as a linear function of some covariates: $\beta_0 + x_{1i}\beta_1 + x_{2i}\beta_2 + \ldots$ where $i$ indexes units of analysis, $x_{1i}$ and $x_{2i}$ are covariates or predictors, and $\Pr(y_i = 1)$ is often denoted as $p_i$. In the GLMM version of logistic regression, $E(y_{it} \mid b_i) = n_{it} p_{it}$ and $\text{logit}(p_{it}) = x_{it}\beta + b_i$ where $y_{it}$ is the binomial-distributed number of visits in CT $i$ on day $t$, $n_{it}$ is the number of members living in that CT on that day, $p_{it}$ is the probability that any patient has had a visit with a diagnosis in the syndrome, $x_{it}$ is a set of covariates measured on CT $i$ at time $t$, $\beta$ is a vector of fixed effects, and $b_i$ is a random effect distributed with mean 0 and variance $\sigma_b^2$ . The model can be used to generate an estimate of $p_{it}$ by inverting the logit.

In the model now in use, we include in $x_{it}$ an intercept, an indicator for 6 days of the week, indicators for 11 months of the year, an indicator of the day as regular or a national holiday, and a linear term for the secular time trend. In each case, the terms contribute significantly to the fit of the model ($p < 0.0001$). The estimates from the model have face validity: the estimated odds of visits for lower respiratory infections are higher in winter months than summer, higher on weekdays than on the weekend, and smaller on national holidays. The test that $\sigma_b^2 = 0$ tests the null hypothesis that all the census tracts are the same, meaning that $p_{it} = p_{jt}$ , for all CT $i$ and $j$. This test is rejected ($p < 0.0001$).

Table 2. Lower respiratory syndrome by census tract, Massachusetts: sample small area report for March 4, 2002[a]

| Population center | Census tract code | Cases in tract | Denominator in this tract | No. of days between counts this extreme[b] |
|---|---|---|---|---|
| Randolph | 250214202 | 4 | 1,232 | 1 |
| Brookline | 250214006 | 2 | 730 | 1 |
| Boston | 250250902 | 1 | 136 | 1 |
| Somerville | 250173507 | 2 | 918 | 1 |
| Boston | 250250304 | 1 | 225 | 1 |

[a]No census tract had an unusual number of new lower respiratory syndrome episodes on that day. The five most extreme tracts are shown, plus all with counts not expected to occur more than once per month. Tracts with most extreme counts are compared with their own history.
[b]Estimated number of days between counts this extreme in any of the 529 census tracts, when data are adjusted for this tract's unique characteristics, as well as month, day of week, holidays, and secular trend.

For example, suppose that the estimated intercept was –8, the estimated effect for April was –0.6, and the estimated effect for Monday was –0.5. Finally, if we are interested in finding $p_{it}$ for a given day in April in a CT with an estimated $b_i$ of 1.1, we omit the secular time trend for simplicity. The estimated $p_{it}$ , $\hat{p}_{it}$ , for any Monday in April in that CT is

$$\text{logit}^{-1}(e^{-8-0.6-0.5+1.1}) = \left(\frac{e^{-8-0.6-0.5+1.1}}{1+e^{-8-0.6-0.5+1.1}}\right) = 0.000335 .$$

The models are applied at the CT level to estimate the period of observation required to expect one count at least as high as those observed in each CT for each syndrome, after the data were adjusted for day of week, holidays, season, secular trend, and the unique characteristics of each CT. This period is also corrected to reflect the fact that each CT is considered on each day. The reported period is the inverse of the expected number of counts this extreme in a day, where 529 tests are performed each day. This is 529* where the last figure is the probability under the model that as many or more cases than were observed on that day will be observed in CT, calculated from the binomial distribution function with $p = p_{it}$ and $n = n_{it}$. The surveillance report (Table 2) shows the five CTs with the longest required period derived from the model, plus all CTs with counts likely to occur only once a month or less

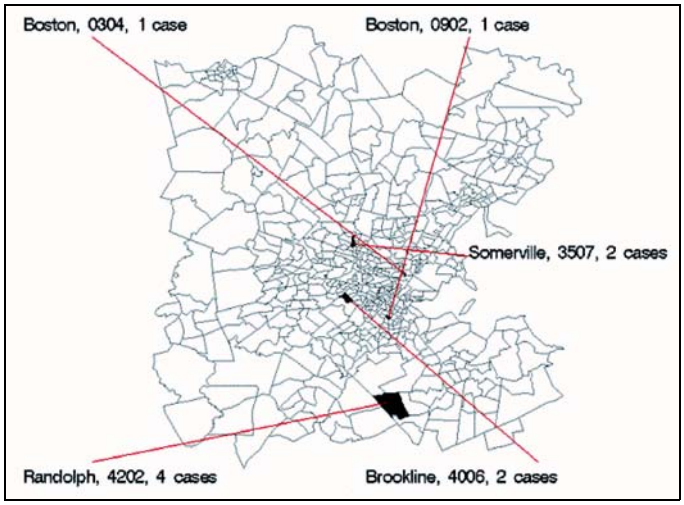


Figure 1. Map of sample small area syndrome counts for Monday, March 4, 2002, showing the five census tracts with the most extreme probability values. Labels show name of town, census tract code (state and county prefixes have been removed), and number of cases for the 24 hours included in the report.

often. We present the model in this fashion so that large numbers are unusual, rather than the smaller-is-more-unusual format of the p-value. In addition, this format has the advantage of being measured in the time scale rather than the probability scale. A map of eastern Massachusetts shows the spatial relationship between CTs highlighted each day (Figure 1).

We also used the model to generate the probability that a count as large or larger than the observed count would be seen over the whole surveillance area, after adjusting for day of week, holidays, season, and census tract variation. This adjustment is done by the same process as for the individual tracts except that the random effects are omitted. These values are also then adjusted on a yearly basis to account for the fact that the probabilities are estimated every day. This estimate is simply the probability that a count as or more extreme as the observed one would be observed in 365 days. All the statistical processing uses automated SAS (18) programs. The web interface was developed by using the Zope web application platform (19), which runs a Python (20) program to rewrite the SAS output files as linked web pages.

### Validation

In the absence of known bioterrorism events, one way of validating the surveillance system is to compare the relationship between the substantial seasonal changes in disease incidence known to occur in the ambulatory-care setting (8) to the seasonal pattern in a reliable and independent source of data such as the hospital system. The lower respiratory syndrome includes a range of diseases (8) commonly associated with admission for an acute illness after a variable prodrome, so this syndrome was chosen for comparison.

Health plan membership is not uniformly distributed throughout the population of Massachusetts (Figure 2). One hundred twenty zip codes were identified in which >100 lower respiratory syndrome episodes were identified in health plan members during 1996–1999; these cases accounted for approximately 70% of all ambulatory lower respiratory syndrome episodes recorded in health plan members.

The weekly numbers of these episodes in health plan members were compared with weekly hospital admissions for all residents (not limited to health plan members) of the same 120 zip codes. Hospital admission data with personal identifiers removed were obtained from the Massachusetts Division of Health Care Finance and Policy for the 3 years ending September 30, 1999. These records included only patients discharged from the hospital, so the final 3 weeks of the hospital admission data were truncated to minimize the "edge effect" from the period when patients may have been admitted but not yet discharged and thus were not included in the available data.

Using the same procedure to group hospital discharge ICD-9 codes as was used for the ambulatory data, we identified all admissions from residents of the 120 zip codes who had a discharge diagnosis in the lower respiratory syndrome group. Hospitalizations were assigned to the date of admission. We compiled the number of ambulatory lower respiratory syndrome episodes and the number of hospital admissions for lower respiratory syndrome for each week for the 3 years ending September 30, 1999. Time-series plots were prepared to compare seasonal patterns in the two independent data sources, and Spearman rank correlations were calculated between weekly hospital admission counts and ambulatory care episodes in the same week, the previous week, and so on up to 6 weeks, by using SAS Proc CORR (18).

## Results

Data from an example of the summary report, one of the syndrome census tract reports, and the corresponding map for March 4, 2002, are shown in Tables 1 and 2 and Figure 1, respectively. The overall counts were all well within model-based expectations for this time of year, so the associated probabilities were all close to 1; at the level of census tracts,

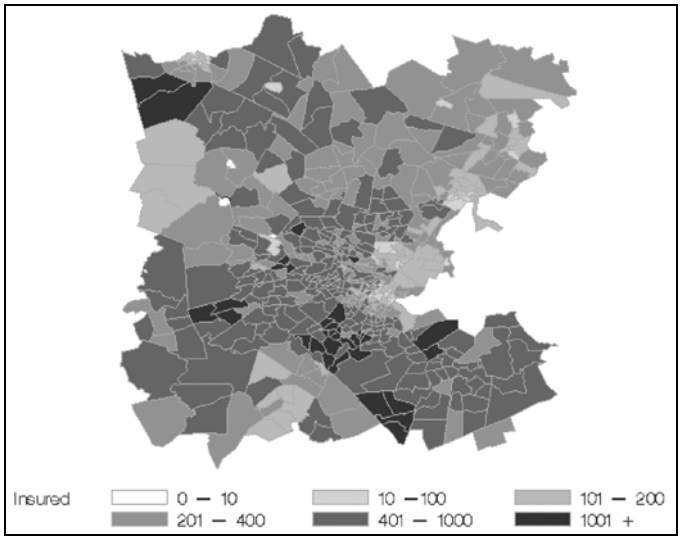


Figure 2. Health plan membership by census tract in eastern Boston. Each census tract contains approximately 4,000 residents.

all counts are common enough to be expected daily. Figure 3 shows daily rates of new episodes of influenza-like illness and lower respiratory syndromes. Day-to-day variation is marked, especially on weekends, as is the expected winter increase in rates. Holidays such as New Year's Day have the lowest rates of reported illness.

The sensitivity of the statistical model in the face of this extreme day-to-day and seasonal variation is illustrated in Table 3. As few as three cases among health plan members may constitute an event predicted by the GLMM to occur less often than once per year, depending on the day of week and the month of the year.

Visual inspection of the weekly counts of episodes in the ambulatory setting compared with hospital admissions shows congruent patterns, including pronounced winter peaks (Figure 4). The data for admissions appear to lag behind data for ambulatory-care visits, most obviously for the winters of 1997 and 1999. Overall, weekly ambulatory-care episodes for lower respiratory illness were highly correlated with hospital admissions over the 3 years examined. The Spearman rank correlation between hospital admissions and ambulatory-care visits during the same week was 0.89. Correlating hospital admissions with ambulatory encounters from the previous week yielded a value of 0.90. Repeating this analysis, increasing the lag by 1 week at a time up to 6 weeks, yielded correlations of 0.92 at 2 weeks, 0.89 at 3 weeks, 0.85 at 4 weeks, 0.80 at 5 weeks, and 0.76 at 6 weeks.

## Discussion

The approach we have taken in the syndrome reporting system is to try to maximize the probability that any "signal" from the earliest stages of a bioterrorism or other public health event can be detected above the "noise" of normal clinical practice. The principal value of a syndromic surveillance system like the one described here is its ability to identify clusters of illness manifest by an unusual number of events, none of which individually differs appreciably from common respiratory, gastrointestinal, or other illnesses. Such nonspecific presentations might be the first sign of a widespread bioterrorism attack. They may also be the only routinely available clinical evidence of other important illness clusters, such as influenza or cryptosporidiosis, for which specific diagnostic tests are typically not performed. Even commonly available tests, such as x-rays, leukocyte counts, and sputum cultures are often not performed for lower respiratory illness with fever in an otherwise healthy patient, so syndromic surveillance can complement surveillance for individual cases of severe or unanticipated illness, which depend on detailed information about history, signs, symptoms, and diagnostic testing. Both syndromic and disease-specific surveillance systems are important components of any complete public health system.

To provide the best possible opportunities for effective intervention, an ideal surveillance system should gather timely, valid, and inexpensive data from a sufficiently large proportion of the population to detect events of interest in the region, and then process and present it to public health personnel in a form that enables efficient decision making. Important elements of such a syndromic surveillance system exist in the automated data generated by health plans and other parts of the health-care delivery system as part of routine operations. The system described here meets many of these criteria because it results from collaboration between academic investigators, health-care providers, and public health officials.

The automated medical records used here are well suited for surveillance of ambulatory-care encounters because the system is deeply integrated into the daily work of all clinicians and it is linked to both the provider payment and the membership systems. Although the data used in this system originate in a complete electronic medical record system, most of the syndromes are defined by diagnosis codes that are also available in other automated systems, including nurse hot lines and increasingly common same-day financial claims processing systems. Thus, several different kinds of data sources could contribute to an integrated surveillance network.

Time-series plots (Figure 4) provide some evidence that the data have validity as a measure of illness in the community, since the seasonal pattern is similar to that of independently collected and validated hospital admission records for

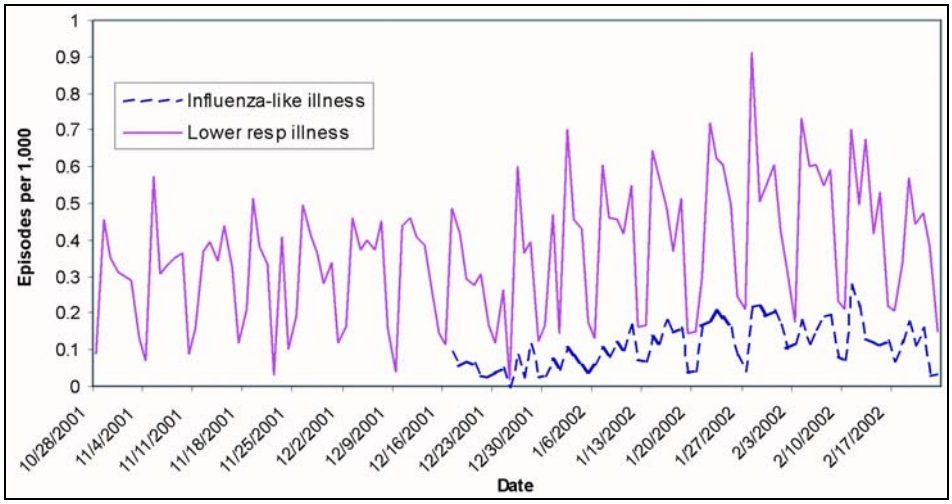


Figure 3. Daily incidence rates of lower respiratory and influenza-like illness after December 17, 2001, showing that within-week variation is substantially greater than seasonal variation.

the same geographic region. The highest correlation, 0.92 at 2 weeks lag, implies that up to 85% of the variability of weekly hospital admission rates is predicted by variation in ambulatory-care admission levels 2 weeks earlier.

Although the principal focus of this system is identifying unusual patterns of apparently common conditions, it also ensures prompt reporting of any encounter with a diagnosis suggestive of a CDC category A bioterrorism agent. In practice, any clinician making one of these diagnoses would be likely to report such a case separately, but there is almost no marginal cost to implement or run this additional surveillance component.

The unadjusted counts and rates for each syndrome (Table 1) may be most useful in responding to a very large and widespread bioterrorism event or identifying expected events such as the advent of influenza in a community. In these cases, statistical refinement is unnecessary because those monitoring the system will see substantially elevated rates. We believe statistical inference will be most useful when the signal from an event is weak or restricted to a small geographic region. Many syndromes have large seasonal fluctuations, such as the well-known winter peak for lower respiratory disease. Individual census tracts also show substantial variability in daily syndrome episode rates, possibly associated with demographic and socioeconomic differences. The statistical model adjusts daily expectations to account for important sources of variation, so those parts of the report based on statistical models take large "expected" seasonal increases in illness into account (e.g., Figure 4). The sensitivity of the resulting system (Tables 1 and 2) in the face of expected variability appears to be much higher than more commonly advocated time-series based analytic approaches for public health surveillance (21).

Daily counts for each syndrome within single census tracts are usually zero, and as few as three to five health plan members affected would be unusual in a typical tract, depending on the month and day of week (Tables 1 and 2). To allow a rapid assessment of the distribution of illness in the region, we highlight the five extreme census tract counts for each syndrome in our daily reports, even though there is nothing unusual in any census tract on most days. An alerting system could easily be triggered when there is a sufficiently unusual cluster for any syndrome. The thresholds can be different for different syndromes, and they can be adjusted to accommodate any desired frequency of alerts. For example, in the absence of a period of heightened alert, public health authorities may wish to be notified when the daily count of syndrome episodes within any census tract attains a level that would only be expected to occur within the entire catchment area once every three or more months. Thus, users will be able to adjust the notification system to suit their needs in terms of the preferred balance of false-positive alerts against the risk of false negatives (no alert in the presence of an actual event of interest). This kind of information, which is being developed as part of this project, could be a useful supplementary source for other public health surveillance systems.

This reporting system includes strong protections of the privacy of individual patients' health records, since routine reports contain only aggregated information. Existing clinical and administrative security protocols that control statutory or other authorized access to confidential patient data will apply when follow-up is requested by public health authorities.

One advantage of this system is that it takes advantage of the experience of ambulatory-care clinicians, who are likely to be among the first to encounter patients during the prodrome of any potential bioterrorism-related or other acute illness. In addition, the system imposes no additional reporting burden on clinicians, thus ensuring unbiased ascertainment of syn-

Table 3. Number of episodes of lower respiratory syndrome that would be expected to occur only once a month and once a year, based on a generalized linear mixed model (GLMM), in a representative eastern Massachusetts census tract[a]

| Month | Day of week | No. needed for once per month event | No. needed for once per year event |
|---|---|---|---|
| January | Monday | 5 | 6 |
| January | Tuesday | 5 | 6 |
| January | Wednesday | 5 | 6 |
| January | Thursday | 5 | 6 |
| January | Friday | 5 | 5 |
| January | Saturday | 4 | 4 |
| January | Sunday | 4 | 4 |
| April | Monday | 4 | 5 |
| April | Tuesday | 4 | 5 |
| April | Wednesday | 4 | 5 |
| April | Thursday | 4 | 5 |
| April | Friday | 4 | 5 |
| April | Saturday | 3 | 4 |
| April | Sunday | 3 | 4 |
| July | Monday | 4 | 5 |
| July | Tuesday | 4 | 4 |
| July | Wednesday | 4 | 4 |
| July | Thursday | 4 | 4 |
| July | Friday | 4 | 4 |
| July | Saturday | 3 | 4 |
| July | Sunday | 3 | 4 |
| October | Monday | 5 | 6 |
| October | Tuesday | 4 | 5 |
| October | Wednesday | 4 | 5 |
| October | Thursday | 4 | 5 |
| October | Friday | 4 | 5 |
| October | Saturday | 4 | 4 |
| October | Sunday | 4 | 4 |

[a]This census tract has 491 health plan members and a random effect of 0.083, illustrating the effect of day of week and month of year for 2002.

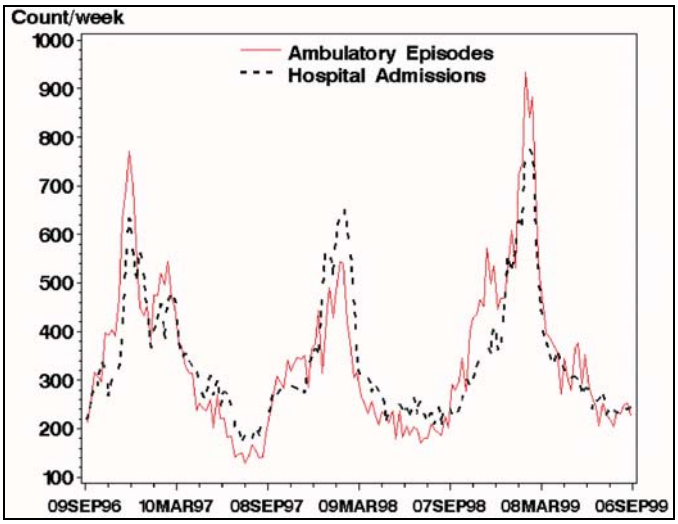


Figure 4. Weekly total ambulatory-care episodes of lower respiratory syndrome (broken line) and hospital admissions for lower respiratory syndrome (solid line) in Massachusetts for the 3 years from September 9, 1996, through September 9, 1999. The eligible population for the hospital data was the entire population of each zip code; the ambulatory care data came from a variable subset of each zip code. As a result, the number of hospital admissions was higher than the number of ambulatory-care episodes for parts of the period shown.

dromes of interest that come to the attention of the practice. The data used here are already being gathered as part of the day-to-day practice of all participating clinicians. There is an initial cost for a system of this type because obtaining the data in a suitable form requires initial programming and testing, but subsequent processing requires relatively little additional expenditure and adds substantial value. All the technology used is widely available and inexpensive.

While any simplification inevitably hides some potentially important detail, we believe that in addition to making the reports more comprehensible, grouping the ICD-9 codes decreases the impact of variation in coding practices. This effect is particularly important since the earliest manifestations of an outbreak may be nonspecific. The fact that syndromic surveillance focuses on unusual counts of common events means that detection of a signal may not be greatly influenced by intensity of diagnostic testing performed, completeness of documentation in the medical record, or variation between physicians or health-care systems in the use of diagnostic terminology or assignment of ICD-9 codes. For example, we have shown that >90% of lower respiratory illness episodes are represented by only three of the 119 ICD-9 diagnosis codes included in the lower respiratory illness syndrome (8). As new ICD coding schemes are adopted, changes to the mapping used to translate code into syndrome will be required, but variation among tens of thousands of discrete individual codes is unlikely to have any major impact at the level of the broad syndromes used in our system.

This emphasis on broad groupings of diagnoses also supports the notion that different data sources, including automated medical records, nurse call centers, and transaction data, might be combined into an integrated surveillance system. Because the focus is on the acute illness that prompts a medical encounter, we expect that the performance characteristics will not be seriously affected by differences between automated data systems, for instance, in the number of diagnoses captured or in the method of assigning diagnosis codes. However, experience with additional systems will be required to elucidate these issues. To the extent that different systems yield similar discrimination of events of interest, it will be possible to integrate them at the regional level, to improve overall sensitivity, and at the national level, to allow coherent surveillance of the entire population.

While many types of data systems can contribute valuable surveillance information, appreciating the added value of more sophisticated data sources is also important. For instance, the availability of temperatures in the automated medical record system described here allows automated surveillance for influenza-like illness. The availability of automated laboratory test results and free text also provides opportunities to detect a wider array of conditions and to improve the specificity of detection of acute illness clusters. For example, anthrax surveillance might be limited to patients with fever and a lower respiratory illness syndrome.

An additional noteworthy feature of surveillance systems such as this one is the fact that they need not cover the entire population to identify at least some clusters of interest. The minimum proportion of the population that must be under surveillance to detect clusters of different sizes has not been determined, but our coverage of 5%–10% of the population of the region appears to provide useful information. Although a small fraction of ambulatory-care practices uses automated medical records, the effective population that would be covered by surveillance systems based on these automated records is substantial, including many of the major population centers in the country. Combining information from these sites with other information sources, such as those maintained by health plans or by hospitals, would rapidly provide at least some monitoring capability for a much larger overall population.

We can suggest additional methods for supplementing a surveillance system that counts syndromes encountered in ambulatory-care visits. Other sources of data, such as school and work absenteeism, over-the-counter medication sales, and even sales of products such as facial tissues and orange juice might contain potentially useful surveillance information. However, whether such data can be cheaply and efficiently gathered and processed and whether the data will yield valid and worthwhile signals remain to be demonstrated.

Many aspects of the current system will be improved with experience. The development of standardized grouping of ICD codes into syndromes is a priority to allow uniform reporting. A great deal of work remains in developing statistical methods capable of detecting different types of illness clusters, ranging from acute, localized increases (for instance, due to release of a toxic chemical agent) to more slowly emerging, widespread conditions, as might be expected from contamination of a water supply. The implementation described here demon-

strates that existing electronic data developed in the course of routine medical care by a wide array of providers and health plans can yield substantial improvements in current public health capabilities for assessment of bioterrorism and other acute illness clusters.

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### Appendix 1

We used this SAS code in fitting the generalized linear mixed model (GLMM) that generates the parameter estimates used in our reports. This SAS code relies on the GLIMMIX macro (17), which has been distributed by SAS (18) since version 6.12.

```
%glimmix (
data=test,
procopt= noclprint covtest ,
stmts=%str(class tract month dayofweek;
model lri/pop=month dayofweek holiday day;
random int/subject=tract solution type=un;),
error=binomial);
```

The data set is structured to contain a row for each day in the historical period for each census tract. In the code, *lri* is the variable that contains the count for census tract *tract* on day *day*. *Pop* contains the number of subjects in the *tract* on that *day*. *Month* is the month of the *day*, *dayofweek* is the day of week of the *day*, and *holiday* indicates whether the *day* is a national holiday. Days are standardized to prevent numerical difficulties with computation.

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# Outbreak of Serogroup W135 Meningococcal Disease after the Hajj Pilgrimage, Europe, 2000

**Jean-François Aguilera,* Anne Perrocheau,† Christine Meffre,‡ Susan Hahné,§ and the W135 Working Group[1]**

The 2000 Hajj (March 15–18) was followed by an outbreak of *Neisseria meningitidis* W135 2a: P1.2,5 in Europe. From March 18 to July 31, 2000, some 90 cases of meningococcal infection were reported from nine countries, mostly the United Kingdom (UK) and France; 14 cases were fatal. Although most early cases were in pilgrims, the outbreak spread to their contacts and then to those with no known pilgrim contact. In France and the UK, the outbreak case-fatality rate was compared with the rate reported from national surveillance. The risk of dying during this outbreak was higher in France and the UK, although the difference was not statistically significant. Prophylaxis for all pilgrims and their household contacts was offered in France; in the UK and other European countries, prophylaxis was recommended only for close contacts. No difference in transmission rates following intervention was detected between France and the UK.

The annual Islamic pilgrimage to Mecca, the Hajj, attracts more than a million pilgrims from many countries worldwide and has been associated with outbreaks of meningococcal disease. The first reported international outbreak of this disease following the Hajj, which was caused by *Neisseria meningitidis* serogroup A, occurred in 1987 (1–3). That epidemic emphasized the potentially high risk of transmission of *N. meningitidis* during the pilgrimage. Before this outbreak, vaccination against *N. meningitidis* serogroup A was required only for pilgrims from sub-Saharan countries to obtain a visa for Saudi Arabia (4). After this outbreak, vaccination became compulsory for all subsequent pilgrims. In 1992, another group A outbreak occurred in Mecca during Umra and Ramadan, but this outbreak was not known to have spread beyond Saudi Arabia (4). Subsequently, the vaccination policy was extended to all Umra visitors.

In March and April 2000, national reference centers (NRC) for meningococci in several European countries detected a sharp rise in isolates of *N. meningitidis* serogroup W135, serotype 2a subtype P1.2,5. This strain, rarely isolated in European countries, belonged to the electrophoretic type 37 (ET-37) clonal complex, which is known to cause hyperendemic disease activity (5). Cases of meningococcal disease caused by serogroup W135 following the Hajj were also reported from Saudi Arabia, the United States, and some countries in North Africa, the Middle East, and Asia (6,7).

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In most European countries, according to national policies, prophylaxis was recommended only for close contacts of cases. In France, as soon as the outbreak was recognized (April 8, 2000, week 14), the Ministry of Health recommended a 2-day course of rifampicin for all pilgrims and their household contacts, with the objectives of preventing cases in pilgrims' households and limiting the spread of the outbreak strain in the population.

We describe the epidemiology of the outbreak in Europe and evaluate the impact of the French intervention.

## Methods

A confirmed case was defined as invasive disease caused by *N. meningitidis* of serogroup W135 2a P1.2,5 or belonging to the ET-37 complex. A probable case was defined as illness in a pilgrim or a pilgrim contact, with either invasive disease due to *N. meningitidis* serogroup W135 of unknown serotype (identified by polymerase chain reaction [PCR] or detection of specific antigens) or with a clinical diagnosis of invasive men-

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ingococcal infection without microbiologic confirmation. Cases included were those with dates of hospital admission from March 18, 2000, until July 31, 2000.

Nonpilgrim case-patients were classified as 1) living in the same household as a pilgrim during the 7 days before the date of onset, 2) being in contact with a pilgrim but not living in the same household during the 7 days before the date of onset, or 3) having no identified contact with a pilgrim.

A questionnaire was sent by e-mail in April 2000 to the National Surveillance Centers in Europe, and interviews of cases were conducted by telephone or in person. For each case, information was anonymously requested on demography, ethnicity, clinical symptoms, medical history, housing situation, meningococcal vaccination history, contact with Hajj 2000 pilgrims, and travel to Saudi Arabia. Results of the microbiologic investigation were obtained from NRC. Additionally, the number of visas delivered for the Hajj 2000 was obtained from Saudi embassies, and information was collected in France on the observance of the specific prophylactic measures for pilgrims and their household contacts.

As cases occurred predominantly in France and the UK, only these two countries were considered in further analysis. The case-fatality rate (CFR) observed in cases from France was compared with the CFR of all *N. meningitidis* infections reported to the national surveillance system in France during 1995–1999. The same comparison was performed for the UK. The probability of dying from the outbreak strain was estimated for France and the UK by using a logistic regression model. In addition to the outcome (death), variables included in the model were age and having an epidemic case versus a national surveillance case. Consecutively, the age-adjusted relative risks (RR) of dying from the epidemic were estimated from the odd ratios by the log link function.

To evaluate the impact of the French control measures implemented on April 8, we compared the number of cases in France with those in the UK before and after April 8 for a) pilgrim and household contacts, and b) out-of-household contacts and those for whom no contact with a pilgrim was identified. The ratio of cases in France after and before the intervention was compared with the same ratio in the UK. An effective intervention would be expected to result in a measure of impact less than one, and vice versa. Confidence intervals were determined by Poisson regression. The p-values were estimated by Fisher's exact test.

The descriptive analysis was carried out with Epi Info (Centers for Disease Control and Prevention, version 6.04c). The logistic regression was performed with Stata version 6.0 (Stata Corporation, College Station, TX). Data on cases reported during 1995–1999 were issued from the Public Health Laboratory Service in the UK and from the Institut de Veille Sanitaire in France.

## Results

From March 18 to July 31, 2000, some 90 cases of serogroup W135 meningococcal disease were ascertained from nine European countries (the UK, France, the Netherlands, Germany, Finland, Sweden, Belgium, Switzerland, and Norway) (Figure 1). Questionnaires were fully completed for 80 cases (89%), and partly completed for 10 cases. Eighty-four isolates (93%) were confirmed as N. meningitidis serogroup W135 serotype 2a subtype P1.2,5. Six probable cases were diagnosed by soluble antigen detection (one case) or PCR (five cases). Microbiologic examination of bacterial isolates was described elsewhere (5).

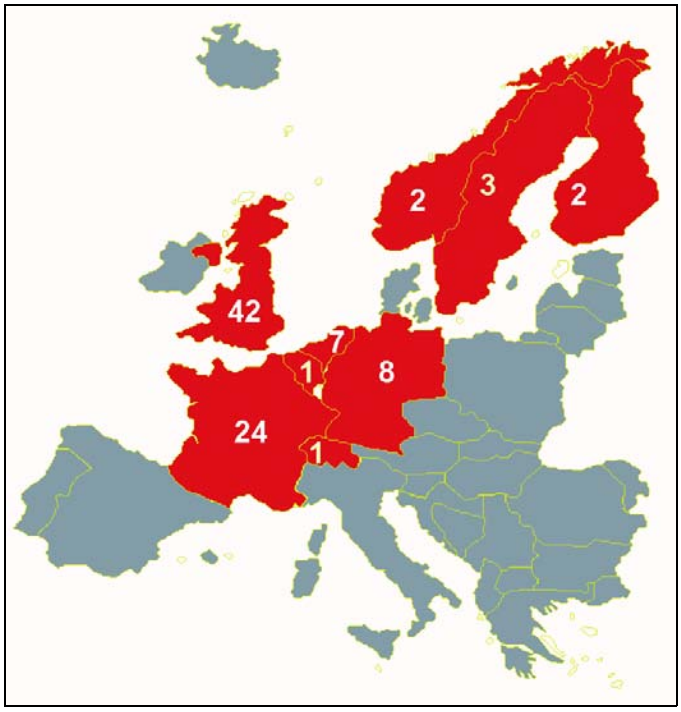


Figure 1. Cases of W135 meningococcal disease reported per country in Europe after Hajj 2000, March 18–July 30, 2000.

The Hajj 2000 was held March 15–18, 2000 (week 11). The peak of the outbreak was rapidly reached in week 14, two weeks after the first return of pilgrims from Mecca. Since that time, the number of cases regularly decreased (Figure 2a). Of 90 cases, 45 (50%) occurred during the first 4 weeks after the first return of pilgrims. In the UK, the first reported cases occurred within 1 week after the Hajj and increased rapidly to a peak of 12 cases in week 14 (Figure 2b). In France, the outbreak started and peaked in week 13 (Figure 2c). For the other countries, cases occurred sporadically during the 4 months after the Hajj.

Twelve cases (13%) were in pilgrims (all vaccinated with vaccine against meningitis A and C before traveling to Mecca), 31 (34%) in household contacts of a pilgrim, and 21 (23%) in contacts outside the household; for 26 cases (29%), no pilgrim contact was identified.

A total of 19,749 pilgrims from the UK and 19,100 from France participated in the Hajj 2000. Eight cases of meningococcal disease occurred in the UK and four in France, giving incidence rates in the pilgrim population of 41 and 21/100,000, respectively. No cases occurred in pilgrims from other

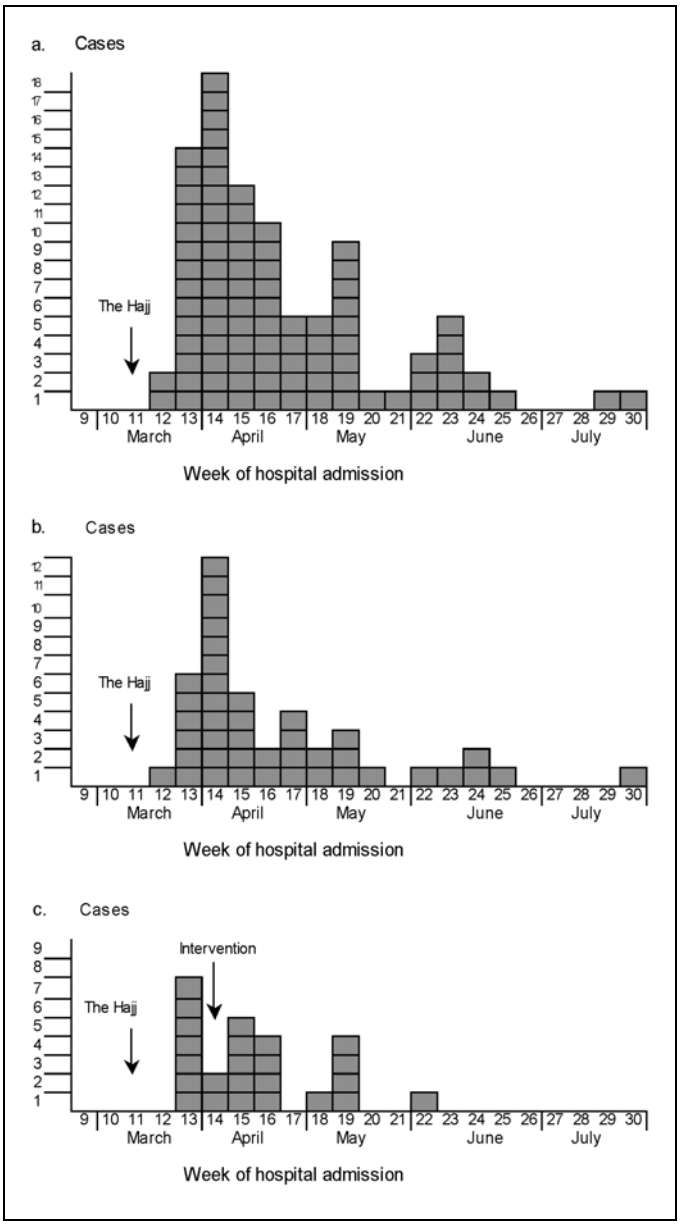


Figure 2. Cases of W135 invasive meningococcal disease by week of hospital admission, March–July 2000: a. Europe (90 cases), b. the United Kingdom (42 cases), and c. France (24 cases).

countries, although Germany had 18,000 pilgrims and the Netherlands had 4,500 pilgrims. For Finland, Sweden, Belgium, Switzerland, and Norway, no data were available on visas delivered.

Pilgrims were affected first (all cases in pilgrims were reported in the 4 weeks after the Hajj): the peak of cases occurred in week 13, household contacts cases peaked in week 14, out-of-household contact cases peaked in week 16, and cases with unknown or no identified contact with a pilgrim peaked in week 19 (Figure 3).

Forty-seven (54%) of the 87 patients whose sex was known were female. Fifty-one (65%) of the 78 nonpilgrim patients were <5 years of age. The median age of the pilgrim patients was 51 years; for nonpilgrims, it was 2 years.

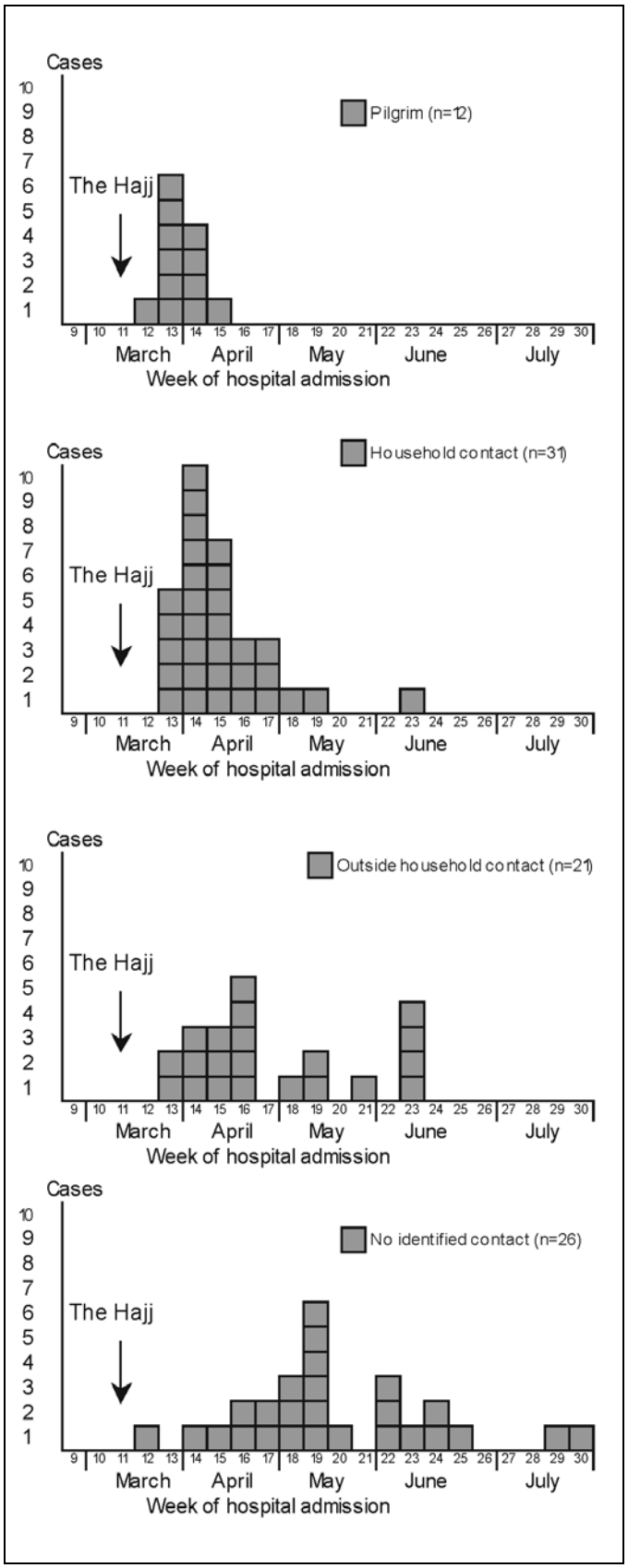


Figure 3. Cases of W135 invasive meningococcal disease, by week of hospital admission and type of contact, Europe, March–July 2000.

Information on clinical features was available for 69 cases, including meningitis (30 cases), septicemia (23 cases), or both (16 cases). Arthritis (six cases), osteomyelitis (one), and pneumonia (one) were also reported from patients with septicemia. Purpura fulminans was reported in 12 cases. Thirteen patients had underlying long-term diseases, but none had preexisting immunodeficiency. In the UK, 75% of patients were of Indian ethnicity; in France, the majority (74%) were North African.

Fourteen patients died (CFR 15.6%, 14/90) (Table 1). In France, 4 (16.7%) of 24 case-patients died, compared with 152 (10.3%) of 1,481) observed for meningococcal disease due to all serogroups of N. meningitidis in France from 1995 to 1999. The age-adjusted RR of dying during the outbreak was 1.26, (95% CI [0.52; 3.06], p=0.63). In the UK, 8 (19.0%) of 42 patients died, compared with 862 (8.3%) of 10,448 observed for meningococcal disease due to all serogroups of N. meningitidis in the UK during1995–1999. The age-adjusted RR was 1.99 (confidence interval 1.02; 2.74, p=0.06). There was no evidence that the RR in France differed from the RR in the UK (p=0.55).

The median length of stay in Saudi Arabia for pilgrim case-patients was 21 days (range 14–40). The median interval between the beginning of the Hajj and the date of onset of disease was 16 days (range 11–29); the median interval between return from the pilgrimage and the onset of disease was 2 days (range 0–8). Among cases with pilgrim contact, the median interval between return of the pilgrim from Mecca and onset of disease was 8 days for household contacts and 6.5 days for out-of-household contacts (p=0.58). Further characteristics of pilgrim cases and cases by type of contact with a pilgrim are shown in Table 2.

Regarding the assessment of the French recommendations, the comparison between France and the UK of the ratio of cases after and before interventions were put in place was 2.43 for pilgrims and their household contacts. This result of >1 yields no evidence that the French measures had a positive impact in preventing cases in pilgrims and their household contacts (p=0.27) (Table 3). The ratio among out-of-household and no known contacts was 0.56, indicating a possible positive impact of the measures in limiting the spread of the outbreak strain, although the result was not statistically significant (p=0.62).

Table 1. Cases of W135 meningococcal disease reported from nine European countries following Hajj, 2000

| | UK (no., % ; n=42) | France (no., % ; n=24) | All other countries (no., % ; n=24) [a] | Total (no., % ; n=90) |
|---|---|---|---|---|
| Sex ratio (M/F) | 0.6 | 0.7 | 1.7 | 0.9 |
| Age distribution | | | | |
| <1 year | 6 (14) | 3 (13) | 5 (21) | 14 (16) |
| 1–4 | 14 (33) | 7 (29) | 14 (58) | 35 (39) |
| 5–9 | 6 (14) | 2 (8) | 0 | 8 (9) |
| 10–19 | 0 | 2 (8) | 1 (4) | 3 (3) |
| 20–49 | 7 (17) | 3 (13) | 1 (4) | 10 (11) |
| 50–65 | 5 (12) | 4 (17) | 0 | 10 (11) |
| >65 | 4 (10) | 3 (13) | 1 (4) | 8 (9) |
| Unknown | 0 | 0 | 2 | 2 |
| No. of deaths | | | | |
| <1 year | 0 | 0 | 0 | 0 |
| 1–4 | 1 | 1 | 2 | 4 |
| 5–9 | 1 | 0 | 0 | 1 |
| 10–19 | 0 | 0 | 0 | 0 |
| 20–49 | 3 | 1 | 0 | 4 |
| 50-64 | 1 | 1 | 0 | 2 |
| >65 | 2 | 1 | 0 | 3 |
| CFR (overall) | 19.0% | 16.7% | 8.3% | 15.6% |

[a]The Netherlands, Germany, Finland, Sweden, Belgium, Norway, and Switzerland.
CFR, case-fatality rate.
UK, United Kingdom.

## Discussion

This report is the first description of a European meningitis outbreak involving nine countries. An international coordination group was set up within 2 weeks after the outbreak was recognized in Europe, followed by an early warning alert to all the European countries. This facilitated information sharing, standardization of the case definition, and implementation of a standardized questionnaire for the investigation. The willingness of participant countries led to a satisfactory completion rate for reports, allowing a precise description of the outbreak throughout Europe. However, case ascertainment may have varied in different countries since some countries identified cases through microbiologic findings only and some through clinical as well as microbiologic findings.

In 1987, an outbreak of meningococcal disease linked to the Hajj was described in several European countries, but the description was limited to pilgrim cases and a few secondary

Table 2. Characteristics of pilgrim and contact cases in Europe following the Hajj, 2000

| | Pilgrims | Household contacts | Outside-of-household contacts | No identified contact |
|---|---|---|---|---|
| No. of cases | 12 | 31 | 21 | 26 |
| Mean age (yrs) | 50.0 | 16.0 | 6.9 | 25.8 |
| Sex ratio (M/F) | 4/8 | 13/18 | 13/8 | 10/13[a] |
| Median no. of bedrooms in household | 3.0 | 2.5 | 2.5 | 2.0 |
| Median no. of rooms in household | 6.0 | 5.0 | 5.5 | 4.0 |
| CFR | 5/12 (41.7%) | 3/31 (9.7%) | 1/18 (5.6%) | 5/21 (23.8%) |

[a] Sex not known for three cases.
CFR, case-fatality rate.

Table 3. Number (and ratio) of cases in United Kingdom (UK) and France before and after a French Ministry of Health recommendation[a]

| | UK (n=42) | | | France (n=24) | | | Measure of impact (Ratio France / ratio UK) |
|---|---|---|---|---|---|---|---|
| | No. of cases | | Ratio | No. of cases | | Ratio | |
| | Before | After | After/before | Before | After | After/before | |
| Pilgrim cases (a) | 8 | 0 | | 3 | 1 | | |
| Household contacts (b) | 9 | 5 | | 4 | 4 | | |
| (a)+(b) | 17 | 5 | 0.3 | 7 | 5 | 0.7 | 2.4 [0.5; 11.1]; p=0.27 |
| Outside-of-household contacts (c) | 2 | 4 | | 2 | 5 | | |
| No identified contact (d) | 0 | 14 | | 0 | 5 | | |
| (c)+(d) | 2 | 18 | 9 | 2 | 10 | 5 | 0.6 [0.1; 4.6]; p=0.62 |
| All cases | 19 | 23 | 1.2 | 9 | 15 | 1.7 | 1.4 [0.5; 3.8];p=0.61 |

[a]April 8, 2000. Ratio is divided by the same ratio in the United Kingdom and its 95% confidence interval.

cases (1–3). In the Hajj 2000 outbreak, the added value of molecular biological investigation, together with the epidemiologic investigation, allowed us to describe a W135 clonal outbreak and the diffusion of this strain from pilgrims to the general population (5). In Sweden, *N. meningitidis* W135 of the same serotype and subtype has been documented since 1979, but pulsed-field gel electrophoresis and the sulfadiazine resistance of the W135 isolates indicate that the outbreak was probably due to a new strain of W135 meningococci (8).

After its description in 1968 and during the 1970s, *N. meningitidis* W135 was considered a minor serogroup, of little clinical importance (9). Only in the early 1980s was this organism described as a fully pathogenic strain, as an important new cause of disease in Europe and the United States and as an emerging cause of meningococcal disease in Africa (10,11). During the 1990s, *N. meningitidis* W135 represented 2.6% to 4% of all reported *N. meningitidis* in the UK, France, and the United States (12–14). The first two cases of meningococcal disease in pilgrims due to W135 associated with the Hajj were described in 1993 in Saudi Arabia in an Indonesian and an American pilgrim (15).

From 1998 to March 2000, fewer than two cases of the W135 2a:P1-5,2 strain were reported yearly in England and Wales (Kaczmarski EB, pers. comm.) and two cases per year in France (Taha MK, pers. comm.). The outbreak strain belonged to the ET-37 complex, which is mainly composed of serogroup C (16). The ET-37 complex has caused hyperendemic disease activity and outbreaks worldwide. It causes disease in clusters and has a higher transmissibility than other strains (5,17,18).

Although the CFRs in cases from France and the UK were high, the age-adjusted RRs of dying during the outbreak were not significantly higher than those observed in the routine surveillance of meningococcal disease due to all serogroup of *N. meningitidis* in these two countries. Thus, the outbreak strain appears to be of similar virulence to *N. meningitidis* serogroups that normally cause meningococcal disease in the UK and France. CFRs have been shown to be linked with age and to increase among very young and older people (19). The initially large number of cases in older people at the beginning of the outbreak might explain this finding.

Methods used to evaluate the impact of the specific control measures implemented in France intended to take into account differences in meningococcal disease incidence rates between the two countries and potential differences in pilgrims' initial carriage of the outbreak strain at their return from Mecca. Since the number of cases in each group (pilgrims, household, out of household, and no identified contact with a pilgrim) was low, the power of the test did not allow identification of the difference of impact between them. Defining other groups could not have allowed conclusions to be drawn, since the number would have also been low. Information collected from the only manufacturer of rifampicin in France indicated that the total number of doses distributed to pharmacies represented only half the doses needed to treat the target group (approximately 100,000 persons living in pilgrims' households), indicating that compliance with the recommendations was low. In addition, all those who were provided treatment may not have taken it effectively, although compliance would not be expected to be a major problem with only 2 days of medication. As of the end of March 2001 in France, no cases were reported in persons who had taken rifampicin and no strain of *N. meningitidis* W135 resistant to rifampicin had been isolated at the NRC. For the prevention of cases among pilgrims and household contacts, the <1 ratio between France and the UK indicated that the measure had no impact in preventing cases in pilgrims and pilgrims' household in France. This might be due to the delay of 2 weeks between the first return of pilgrims and the release of the measure, an interval during which transmission of the pathogenic strain occurred inside the pilgrims' households.

The absence of significant impact of the measure to limit the diffusion of the pathogenic strain to the out-of-household contacts and persons with no contacts identified may be

explained either by the very small number of cases considered or by potential misclassification. Cases in the general population may also have been underestimated in comparison with the likely high case ascertainment in pilgrims. However, such underestimates are unlikely since virtually all invasive strains of *N. meningitidis* are sent to the reference laboratory in France and the UK. However, data for cases of W135, 2a P1.2,5 obtained from national reference laboratories in France and the UK for September 2000–February 2001 indicated that 13 cases were reported in the UK and 9 in France, suggesting that there was no long-lasting effect of the measure and that immunity to the strain was probably increasing in the population (20). In the UK, carriage studies showed that this strain was still circulating within the Muslim community (Stuart JM, pers. comm.). The results of the measures implemented in France do not allow us to draw conclusions for use of mass prophylaxis in the future, mainly because of the small number of cases in our study.

Following this outbreak, France and the UK, among other countries, recommended quadrivalent vaccine for travelers to the Hajj 2001 (21,22; pers. comm., Secrétariat du Conseil Supérieur d'Hygiène Publique de France). Subsequent quadrivalent vaccine coverage was estimated to be 47% and 65% in the UK and in France, respectively. Another outbreak of meningococcal disease caused by *N. meningitidis* W135 2a P1.2,5 occurred in Hajj pilgrims and their contacts in 2001; most cases were from Saudi Arabia and the UK. During the period March 28–June 29, 2001, 10 cases of meningococcal disease due to W135 2a P1.2,5 were reported to the NRC in France (0 deaths) and 25 in the UK (8 deaths) (23–25). Since May 2001, quadrivalent vaccine is now a requirement for all pilgrims to future Hajj pilgrimages (26).

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# Genetic Characterization of Hantaviruses Transmitted by the Korean Field Mouse (*Apodemus peninsulae*), Far East Russia

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In an epizootiologic survey of 122 rodents captured in Vladivostok, Russia, antibodies positive for hantavirus were found in *Apodemus peninsulae* (4/70), *A. agrarius* (1/39), and *Clethrionomys rufocanus* (1/8). The hantavirus sequences identified in two seropositive *A. peninsulae* and two patients with hemorrhagic fever with renal syndrome (HFRS) from the Primorye region of Far East Russia were designated as Solovey and Primorye, respectively. The nucleotide sequences of the Solovey, Primorye, and Amur (obtained through GenBank) sequences were closely related (>92% identity). Solovey and Primorye sequences shared 84% nucleotide identity with the prototype Hantaan 76-118. Phylogenetic analysis also indicated a close relationship between Solovey, Primorye, Amur, and other viruses identified in Russia, China, and Korea. Our findings suggest that the Korean field mouse (*A. peninsulae*) is the reservoir for a hantavirus that causes HFRS over a vast area of east Asia, including Far East Russia.

Currently, at least 20 serotypes and genotypes of the *Hantavirus* genus (family: *Bunyaviridae*) have been identified worldwide. Rodents are the natural reservoir for hantaviruses, although one virus strain has been isolated from the house shrew (*Suncus murinus*), an insectivore (1). A unique characteristic of hantaviruses is the close association between the virus type and its natural reservoir (2).

Hantaviruses cause two forms of human disease, hemorrhagic fever with renal syndrome (HFRS), and hantavirus pulmonary syndrome (HPS); human infection occurs after the inhalation of aerosolized rodent excreta. HFRS is manifested as high fever, renal dysfunction, and hemorrhage; HPS is characterized by an acute progressive pulmonary edema and a fatality rate of about 40%. Among the hantaviruses that cause HFRS in Eurasia are *Hantaan virus* (HTNV), *Seoul virus* (SEOV), *Puumala virus* (PUUV), and *Dobrava-Belgrade virus* (DOBV) (3), which are carried by the striped field mouse (*Apodemus agrarius*), Norway rat (*Rattus norvegicus*) and black rat (*R. rattus*), bank vole (*Clethrionomys glareolus*), and yellow-necked field mouse (*A. flavicollis*), respectively. DOBV was also found in *A. agrarius* in Europe (4,5). *Sin Nombre virus* (SNV), *New York virus* (NYV), *Black Creek Canal virus* (BCCV), *Bayou virus* (BAYV), *Andes virus* (ANDV), and other related viruses cause HPS in the New World and are carried by the deer mouse (*Peromyscus maniculatus*), white-footed mouse (*P. leucopus*), cotton rat (*Sigmodon hispidus)*, marsh rice rat (*Oryzomys palustris),* and *Oligoryzomys longicaudatus*, respectively (2,6). Although the known genotypes and serotypes have increased in number with advances in the knowledge of epidemiology and epizootiology of hantavirus infection (2), some still-unidentified hantaviruses carried by specific rodent hosts may exist. HFRS is generally known to be endemic to Far East Russia. However, the genetics of hantaviruses that are pathogenic for humans are not well defined. Reed voles (*Microtus fortis)* in Far East Russia were found to harbor two novel hantaviruses, *Khabarovsk virus* (KHAB) and Vladivostok virus (7,8). Another hantavirus, *Topografov virus* (TOPV), was isolated from brown lemmings (*Lemmus sibiricus*). The correlation between these three viruses and their pathogenicity for humans are not yet known (9).

A recent study reported two novel hantaviruses, designated as Amur (AMR) and Far East (FE), that were identified from HFRS patients in Far East Russia (10). The natural reservoir of AMR genotype seems to be *A. peninsulae*, according to a recent study on nucleotide sequence comparisons by Yashina et al. (11).

In 1999, we carried out an epizootiologic survey in a suburb of Vladivostok, Russia, to determine the characteristics of hantaviruses circulating in Far East Russia and to examine the possibility that *A. peninsulae* is a carrier of pathogenic hantaviruses. We detected antibodies to hantaviruses in *A. penin-*

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*sulae*, and the viral genome characteristics were extremely similar to the newly identified genotype, AMR (10). Using phylogenetic analysis to characterize the sequences of viruses identified from HFRS patients and *A. peninsulae*, we were able to corroborate the assumption of Yashina et al. (11). We also found that *A. peninsulae*-related viruses are pathogenic for humans and are distributed over a large area of east Asia that includes Far East Russia.

## Materials and Methods

We collected sera and organs from wild rodents captured during 1999. We also collected sera and autopsy materials from HFRS patients in two rural villages in the Primorye region of Russia, located 400 km and 600 km from Vladivostok.

Rodent sera were screened for antibodies to HTNV and PUUV or both by indirect immunofluorescent antibody assay (IFA). Vero E6 cells infected with the Hantaan 76-118 strain of HTNV or the Sotkamo strain of PUUV were used as antigen slides. Diluted sera (1:16 and 1:64) were spotted onto the antigen slides and incubated at 37°C for 1 h. After three washes with phosphate-buffered saline (PBS), protein G-conjugated fluorescein isothiocyanate (FITC) (Zymed Laboratories, Inc., San Francisco, CA) was spotted onto the slides. After incubation at 37°C for 1 h, the slides were washed and observed by fluorescence microscopy. Scattered, granular fluorescence in the cytoplasm of infected Vero E6 cells was considered a positive reaction. Antibodies in HFRS patient sera were detected by the same protocol, except for the substitution of FITC-conjugated antihuman immunoglobulin (Ig) G (ICN Pharmaceuticals, Inc., Aurora, OH).

Total RNA was extracted from lung tissues of seropositive *A. peninsulae* with Isogen (Nippon Gene Co., Ltd., Osaka, Japan), which is based on the acid guanidium-phenol-chloroform technique, according to manufacturer's instructions. Similarly, total RNA was extracted from lung, liver, kidney, spleen, and brain tissues of HFRS patients. Reverse transcription (RT) was carried out at 42°C for 30 min by using Superscript II and random primer (Gibco-BRL, Rockville, MD). Full-length S segments were amplified with Platinum Taq (Gibco-BRL) and HTNV–full S primer for 30 polymerase chain reaction (PCR) cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 2 min. Amplification of M segments was identical to that of S segments, except for the use of M genome-specific primers (Table 1). Part of the M segment (232 nucleotides) and the entire S segment (except for the 5′ and 3′ ends) were sequenced with primers specific for HTNV or SEOV or both. Amplification of the partial M segment was achieved only with nested PCR. The PCR-amplified products were separated by using a Rapid Gel Extraction kit (Gibco-BRL) according to the manufacturer's instructions. Purified DNA fragments were cloned into the PCR 2.1 vector provided in the TA cloning kit (Invitrogen Corporation, Carlsbad, CA). The ligated products were transformed into Top 10 competent cells (Invitrogen Corporation) and purified with a Miniprep kit (QIAGEN GmbH, Hilden, Germany). DNA sequencing was performed with the ABI-PRISM Dye Terminator Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 373-A genetic analyzer.

Table 1. Primers used for reverse transcription-polymerase chain reaction and/or sequencing of S and M genome segments of hantaviruses

| Gene | Primer name | Primer sequence (5′–3′) | Position |
|---|---|---|---|
| S segment | M13 Fw | ctggccgtcgttttac | |
| | PEN 215 S Fw | gaattgaaagacaattggc | 215–233 |
| | KPS3[a] | tc(a/c)agcatgaaggc (a/t)gaagagat | 592–703 |
| | PEN 780 SFw | acagaggcaggcagctttag | 780–799 |
| | PEN 1042 S Fw | gcaggatatgcggaatacaa | 1042–1061 |
| | HTNV 1390 S Fw | attgcactattattatcagg | 1390–1409 |
| | HTNV Full S | ttctgcagtagtagtag(t)a (g)ctccctaa | |
| | PEN180 S Rv | ttccctgtctgttaatgctc | 180–199 |
| | PEN 585 S Rv | tgggcaaggacacatagaga | 585–604 |
| | PEN 946 Rv | atgatggtgactcgatgtct | 946–965 |
| | PEN 1160 S Rv | gttgtattcccattgactgt | 1160–1179 |
| | HTNV 1493 SRv | cacccacaacggattaactg | 1493–1512 |
| | M13 Rv | caggaaacagctatgac | |
| M segment | HS1[a] | ac(a/c)tgtca(c/a)tttgg (a/t)gaccc | 2636–2655 |
| | HS2[a] | tcaca(g/a)gcctttattga(g/t)gt | 3072–3091 |
| | HS3[a] | t(tc)aggaa(ga)aaatg (tc)aactttgc | 2715–2736 |
| | HS4[a] | acacc(a/t)gaaccccaggc(a/c)cc | 3000–3019 |
| | M13 Fw | ctggccgtcgttttac | |
| | M13 Rv | caggaaacagctatgac | |

[a]Primers designed by Yashina et al.

We used the ClustalX program package (version 1.81; available from: URL: ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) to generate the phylogenetic trees by using the neighbor-joining method with 1000 bootstrap replicates. Hantavirus sequences used in the comparisons were obtained from GenBank. The S and M genome sequences used in this study are listed in Table 2.

Formalin-fixed lung, liver, kidney, and brain tissues from an HFRS patient who died of acute renal failure were observed under light microscopy and subjected to immunohistochemical analysis with monoclonal antibodies against Hantaan virus.

## Results

We carried out the epizootiologic survey on 122 rodents captured in a suburb of Vladivostok; results of serologic screening of rodent sera by IFA are shown in Table 3. Identified rodent species included (70) *A. peninsulae*, (39) *A. agrarius*, (8) *C. rufocanus*, (3) *M. fortis*, and (2) *Tamias sibiricus*. Screening by IFA showed that one *A. agrarius* (2.5%), four *A. peninsulae* (5.7%), and one *C. rufocanus* (12.5%) had antibodies to HTNV or PUUV or both. HTNV-antibody titers ranged from 1:32 to 1:512. All the seropositive rodents, except for *C. rufocanus*, lacked antibody against PUUV (Table 4). Lung tis-

Table 2. Hantavirus sequences used in this study[a]

| Virus type | Strain | Source | Country | | Accession nos. | | References |
|---|---|---|---|---|---|---|---|
| | | | Region | Location | M | S | |
| HTNV | SL/AP61/1999 | *Apodemus peninsulae* | Far East Russia | Solovey | AB071185 | AB071183 | This report |
| | SL/AP63/1999 | *A.peninsulae* | Far East Russia | Solovey | AB071186 | AB071184 | This report |
| | PRI/H1/2000 | Human | Primorye | Cavalerovo | AB071187 | —[b] | This report |
| | PRI/H2/2000 | Human | Primorye | Cavalerovo | AB071188 | — | This report |
| | AMR/680 | *A.peninsulae* | Far East Russia | Khabarvosk | AF332571 | — | 11 |
| | AMR/1166 | *A.peninsulae* | Far East Russia | Khabarvosk | AF332569 | — | 11 |
| | AMR/1169 | *A.peninsulae* | Far East Russia | Khabarvosk | AF332570 | — | 11 |
| | AMR/4234 | Human | Far East Russia | Amursk | AF172422 | — | 10 |
| | AMR/4309 | Human | Far East Russia | Amursk | AF172423 | — | 10 |
| | AMR/4313 | Human | Far East Russia | Korphovsky | AF172424 | — | 10 |
| | H8205 | Human | China | — | AB030232 | — | — |
| | HTNV261 | — | China | Heilongjiang | — | AF252259 | — |
| | Z10 | Human | China | Zhejiang | AB027076 | AB027108 | 12 |
| | Chen4 | Human | China | Anhui | — | AB027101 | 12 |
| | Maaji1 | *A. agrarius* | Korea | — | — | AF321094 | Lee PW [c] |
| | Maaji-2 | Human | Korea | — | — | AF321095 | Lee PW [c] |
| | HTN 76-118 | *A. agrarius* | South Korea | — | M14627 | M14626 | 13,14 |
| | Q32 | — | China | Guizhou | — | AB027097 | 12 |
| | HV114 | *A. agrarius* | China | Hubei | L08753 | AB027110 | 12,15 |
| | A9 | *A. agrarius* | China | Jiangsu | AF035831 | — | 16 |
| | Hojo | Human | South Korea | — | D00376 | — | 17 |
| | FE/7866 | Human | Far East Russia | Razdolnoye | AF172439 | — | 10 |
| | NC167 | *Niviventer confucianus* | China | Anhui | AB027115 | AB027523 | 12 |
| | H3 | Human | China | Hubei | — | — | 18 |
| | H5 | Human | China | Heilongjiang | — | — | 18 |
| | A3 | *A. agrarius* | China | Zhejiang | AB027055 | — | 12 |
| | B78 | Human | China | Shandong | AB027056 | AB027093 | 12 |
| | Q36 | *A. agrarius* | China | Guizhou | AB027057 | AB027094 | 12 |
| | Q7 | *A. agrarius* | China | Guizhou | AB02058 | AB027095 | 12 |
| | Q20 | *A. agrarius* | China | Guizhou | AB027059 | AB027096 | 12 |
| | Niongxia-A | *A. agrarius* | China | Niongxia | AB027060 | — | 12 |
| | Q10 | *A. agrarius* | China | Guizhou | AB027062 | AB027098 | 12 |
| | A16 | *A. agrarius* | China | Sanxi | AB027063 | AB027099 | 12 |
| | Q37 | *A. agrarius* | China | Guizhou | AB027064 | AB027100 | 12 |
| | Q33 | *A. agrarius* | China | Guizhou | AB027065 | AB027102 | 12 |
| | Bao9 | *A. agrarius* | China | Heilongjiang | AB027066 | AB027103 | 12 |
| | Jiang13 | *A. agrarius* | China | Heilongjiang | AB027067 | AB027104 | 12 |
| | Bao14 | *A. agrarius* | China | Heilongjiang | AB027068 | AB027105 | 12 |
| | Bao10 | *A. agrarius* | China | Heilongjiang | AB027069 | AB027106 | 12 |
| | Lee | Human | South Korea | — | D00377 | — | 17 |
| | 62HTNV | — | — | — | AB027070 | — | 12 |
| | 6B | — | — | — | AB027071 | — | 12 |
| HTNV | Vaccine | — | — | — | AB027072 | — | 12 |
| | H2 | — | North Korea | — | AB027073 | AB027107 | 12 |
| | HN26-L | *A. agrarius* | China | Hainan | AB027074 | — | 12 |
| | Luyao | Human | China | Shandong | — | AB027109 | 12 |
| | B659 | Human | China | Shandong | S72339 | — | 18 |
| | Hu | Human | China | Hubei | AB027077 | AB027111 | 12 |

Table 2. (continued) Hantavirus sequences used in this study[a]

| Virus type | Strain | Source | Country | | Accession nos. | | References |
|---|---|---|---|---|---|---|---|
| | | | Region | Location | M | S | |
| | Q83 | — | — | Guizhou | AB027078 | — | 12 |
| | B256 | — | — | — | AB027079 | AB027112 | 12 |
| | Thailand | *Bandicota indica* | Thailand | — | L08756 | — | — |
| | Topografov | *Lemmus sibericus* | Far East Russia | Siberia | AJ011647 | — | 9 |
| SEOV | L99 | *Rattus losea* | China | Jiangxi | AF035833 | AF288299 | — |
| | SR11 | *R. norvegicus* | Japan | Sapporo | M34882 | M34881 | 19 |
| | Gou3 | *R. rattus* | China | Zhejiang | AB027521 | AB027522 | 12 |
| | NM39 | *R. norvegicus* | China | Neimeng | AB027080 | — | 12 |
| | HB55 | Human | China | Henan | AF035832 | — | 17 |
| | Wan | Human | China | Jiangsu | AB027081 | — | 12 |
| | J12 | Human | China | Jieling | AB027082 | — | 12 |
| | Henan94 | *R. norvegicus* | China | Henan | AB027083 | — | 12 |
| | Shanxi | — | — | — | AB027084 | — | 12 |
| | HN71-L | *R. norvegicus* | China | Hainan | AB027085 | — | 12 |
| | Guang199 | — | — | — | AB027086 | — | 12 |
| | Beijing-Rn | *R. norvegicus* | China | Beijing | AB027087 | — | 12 |
| | c3 | Human | China | Hebei | AB027088 | — | 12 |
| | Hebei4 | *Cricetulus barabensis* | China | Hebei | AB027090 | — | 12 |
| | SD227 | — | China | Shangdong | AB027091 | — | 12 |
| | SD10 | *R. norvegicus* | China | Shangdong | AB027092 | — | 12 |
| | Hbei1 | Human | China | Hubei | S72343 | — | 17 |
| | Seoul | *R. norvegicus* | South Korea | — | S47716 | — | 20 |
| | Tchoupitoulas | *R. norvegicus* | North America | — | U00473 | — | 21 |
| | B-1 | *R. norvegicus* | Japan | — | X53861 | — | 22 |
| | Girard Point | *R. norvegicus* | North America | — | U00464 | — | — |
| DOBV | DOB/SLOV | *A. flavicollis* | Slovenia | — | L33685 | L41916 | 23 |
| | DOB/SAA | *A. agrarius* | Estonia | — | AJ009774 | AJ009773 | 4 |
| SNV | SNV | *Peromyscus maniculatus* | North America | — | L25783 | L25784 | 24 |
| PUUV | PUU/Sot | *Clethrionomys glareolus* | Finland | — | X61034 | — | 25 |
| | Kamiiso | *C. rufocanus* | Japan | Kamiiso | AB011631 | — | 8 |
| KHAB | Khabarovsk | *Microtis fortis* | Far East Russia | Khabarvosk | AJ011648 | — | 9 |

[a]Abbreviations used: HTNV and HTN, *Haantan virus*; SL, Solovey; PRI, Primorye; AMR, Amur; SEOV, *Seoul virus*; DOB and DOBV, *Dobrava-Belgrade virus*; SLOV, Slovenia; SAA, Saarema; SNV, *Sin Nombre virus*; PUUV, *Puumala virus*; and KHAB, *Khabarovsk virus.*
[b] —, not reported/not used in this study.
[c]Pers. comm.

sues from seropositive *A. peninsulae* were subjected to RT-PCR to amplify the virus genomes. Two of the four rodents with high IFA titers to HTNV (1:256 and 1:512) were positive by PCR for both the S and M segments of hantavirus.

We obtained the clinical histories of two fatal cases of HFRS in the Primorye region. The patients, who lived in villages 400 km and 600 km from Vladivostok, died 8–13 days after the onset of illness; gastrointestinal bleeding and acute renal failure were the causes of death. Serologic screening showed that both patients were positive for hantaviral antibodies. Antibody titers to HTNV and SEOV were apparently higher than to PUUV. We used lung, liver, kidney, spleen, and brain tissues of these HFRS patients for RT-PCR analysis; the lung and kidney tissues of patient no. 1 and the spleen tissue of patient no. 2 were positive for hantaviral M segment.

To examine the histopathologic changes in HFRS patients, we used light microscopy to examine sections of formalin-fixed lung, liver, kidney, spleen, and brain tissues from patient no. 2, who had died of acute renal failure (Figure 1). The kidney was the only tissue that showed the recognizable histopathologic changes. Salient changes included interstitial edema with mild infiltration of mononuclear cells (Figure 1, small arrow) and degeneration of renal tubules (Figure 1, large arrow) in the cortex (Figure 1, A). Although proteinaceous casts and exudates were observed in the lumina of renal tubules (Figure 1, arrowhead), there were no apparent glomerular changes. In addition, a prominent well-defined necrotic lesion (Figure 1, asterisk) was noted in the medulla (Figure 1, B). Viral antigens were not detected in these specimens by using monoclonal antibodies to HTNV.

Table 3. Serologic screening by immunofluorescent antibody assay for *Haantan virus* and *Puumala virus* antibodies in rodents, Vladivostok, Russia[a]

| Rodent species | No. of sera tested | Positives by IFA (%) | |
|---|---|---|---|
| | | HTNV | PUUV |
| *Apodemus peninsulae* | 70 | 4(5.7) | 0 |
| *A. agrarius* | 39 | 1(2.5) | 0 |
| *Clethrionomys rufocanus* | 8 | 1(12.5 | 1(12.5) |
| *Microtus fortis* | 3 | 0 | 0 |
| *Tamias sibiricus* | 2 | 0 | 0 |
| Total | 122 | 6(4.9) | 1(0.8) |

[a]Abbreviations used: IFA, immunofluorescent antibody assay; HTNV, *Haantan virus*; PUUV, *Puumala virus*.

The entire S segments of the viruses from two seropositive *A. peninsulae* were amplified and sequenced. We designated these segments as Solovey/AP61/1999 and Solovey/AP63/1999 based on the name of the village closest to the survey point, the rodent species from which the sample was taken, and the year in which the epizootiologic survey was done. We compared the coding regions of these sequences with those of other hantaviruses (Table 5). The S segments of the two Solovey sequences had 99.0% and 98.8% identities in nucleotide and amino acid sequences, respectively. Solovey sequences and Hantaan viruses had 78.2%–84.5% nucleotide sequence identity and 86.7%–93.3% amino acid sequence identity, regardless of their source or geographical origin. Lower nucleotide sequence identities were seen than in Solovey sequences and other viruses: DOBV (73.6%), SEOV (73.9%), and SNV (63.9%).

To explore the genetic diversity of hantaviruses identified in *A. peninsulae* in more detail, we sequenced the partial M segment of the G2 region (232 nt). We also sequenced the partial M segments of genetic lineages identified in the two HFRS patients from the Primorye region, designated as Primorye/H1/2000 and Primorye/H2/2000. The M segment of Solovey and Primorye sequences were compared with those of other hantaviruses (Table 6). Nucleotide sequence identities among these sequences were between 92.2% and 98.2%; amino acid sequence identities were almost identical (98.7%–100%). We also compared the M segment sequences of Solovey and Primorye with those of AMR genetic lineage, recently identified in HFRS patients and *A. peninsulae* in Far East Russia (10,11). The nucleotide and amino acid identities between Solovey, Primorye, and AMR lineages were 91.3%–98.3% and 93.5%–98.7%, respectively. The M segment sequences of Solovey, Primorye, and AMR lineages were compared with that of H8205, isolated from an HFRS patient in China. In this case, the nucleotide sequence identities were 93.5%–96.1%, and the amino acid sequence identities were 94.8%–100%. Lower nucleotide identities were seen with HTNV (78.8%–86.2%), SEOV (79.3%–81.4%), and DOBV (75.8%–77.1%). This high level of sequence identity among Solovey, Primorye, AMR, and H8205 sequences suggests that some patients acquired the infection from the Korean field mouse (*A. peninsulae*) in Far East Russia and China. Our results also suggest that this

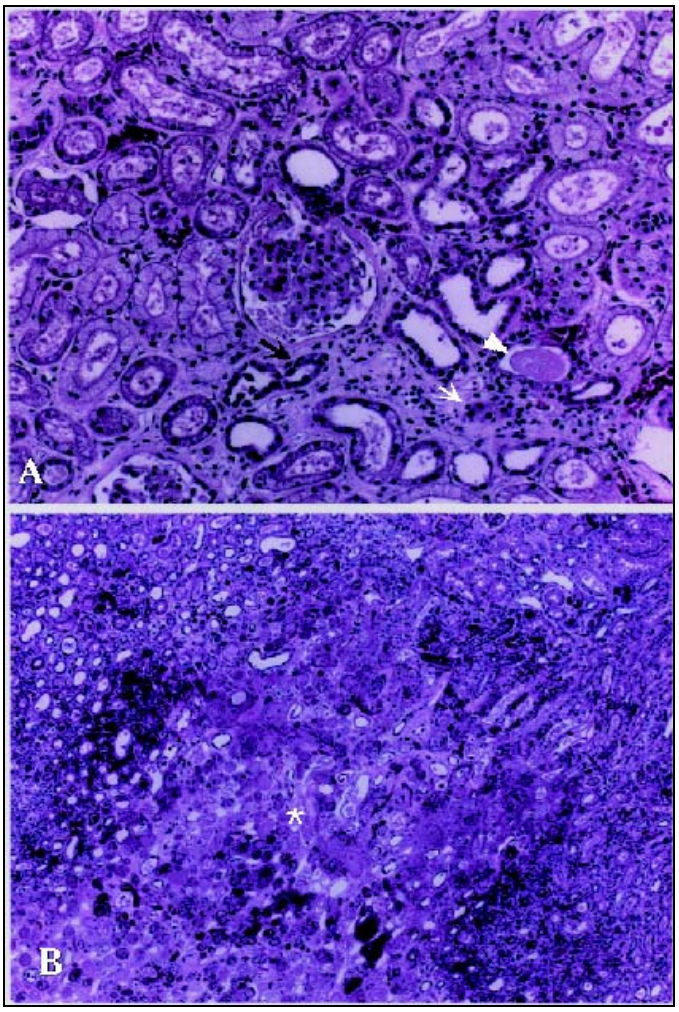


Figure 1. Histopathologic changes in kidney tissue from a patient with hemorrhagic fever with renal syndrome, Primorye region. Changes include interstitial edema with mild infiltration of mononuclear cells (small arrow) and degeneration of renal tubules (large arrow) in cortex. Proteinaceous casts and exudate (arrowhead) are seen in lumina of renal tubules (A). No apparent glomerular changes. Most prominent change in the medulla is well–defined necrotic lesion (asterisk) (B).

Table 4. *Haantan virus* and *Puumala virus* antibody titers determined by immunofluorescent antibody assay and polymerase chain reaction results

| Species | Sample number | IFA antibody titer | | PCR |
|---|---|---|---|---|
| | | HTNV | PUUV | |
| *Apodemus peninsulae* | 47 | 256 | <16 | -[b] |
| *A. peninsulae* | 61 | 512 | <16 | +[c] |
| *A. peninsulae* | 63 | 256 | <16 | + |
| *A. peninsulae* | 74 | 64 | <16 | - |
| *A. agrarius* | 10 | 32 | <16 | NA |
| *Clethrionomys rufocanus* | 32 | 256 | 256 | ND |

[a]Abbreviations used: HTNV, *Haantan virus*; PUUV, *Puumala virus*; IFA, immunofluorescent antibody assay; PCR, polymerase chain reaction; NA, not available; ND, not done.
[b]-, negative.
[c]+, positive.

Table 5. Comparison of nucleotide (open reading frame) and amino acid of S genome between those from *Apodemus peninsulae* and other hantaviruses[a]

| | Nucleotide and amino acid identities %[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL/AP61 | SL/AP63 | HTNV261 | Z10 | Chen4 | Maaji-1 | HTNV 76-118 | Q32 | NC1167 | SR11 | GOU3 | Dob/Slo |
| SL/AP61 | | **99.0** | 84.5 | 83.5 | 83.4 | 82.9 | 82.7 | 82.3 | 78.3 | 73.7 | 73.7 | 72.9 |
| SL/AP63 | **98.8** | | 84.2 | 83.5 | 83.4 | 82.9 | 82.8 | 81.5 | 78.2 | 73.9 | 73.8 | 72.2 |
| HTNV261 | 91.9 | 91.5 | | 85.6 | 85.7 | 83.0 | 88.6 | 84.7 | 78.9 | 74.1 | 73.6 | 72.6 |
| Z10 | 91.9 | 91.5 | 92.9 | | 89.1 | 83.6 | 85.9 | 87.5 | 79.8 | 75.3 | 74.2 | 73.3 |
| Chen4 | 93.0 | 92.5 | 93.2 | 96.2 | | 82.8 | 85.8 | 90.3 | 78.7 | 73.2 | 74.2 | 73.4 |
| Maaji-1 | 91.5 | 90.8 | 90.8 | 91.3 | 93.0 | | 82.9 | 82.1 | 78.2 | 74.2 | 73.0 | 74.2 |
| HTNV76-118 | 92.2 | 91.5 | 94.9 | 92.9 | 93.7 | 91.0 | | 84.4 | 78.2 | 74.6 | 73.8 | 74.0 |
| Q32 | 92.7 | 92.3 | 93.7 | 94.4 | 96.0 | 91.8 | 93.2 | | 79.1 | 73.1 | 74.3 | 73.8 |
| NC167 | 87.2 | 86.7 | 85.3 | 85.8 | 85.3 | 84.8 | 86.9 | 85.1 | | 75.3 | 73.6 | 72.7 |
| SR11 | 75.0 | 74.5 | 74.1 | 73.9 | 74.6 | 74.3 | 74.8 | 74.1 | 77.2 | | 87.8 | 73.7 |
| GOU3 | 75.7 | 75.5 | 75.0 | 74.8 | 76.2 | 74.3 | 74.8 | 76.7 | 76.7 | 91.5 | | 73.1 |
| Dob/Slo | 76.4 | 76.4 | 76.8 | 75.7 | 77.6 | 76.6 | 75.5 | 77.2 | 76.0 | 73.1 | 73.1 | |

[a]Values in bold show the close identities between the two Solovey sequences. Abbreviations used: SL, Solovey; HTNV, Haantan virus; Dob, Dobrova; Slo, Slovenia.
[b]Values above the diagonal and to the right show nucleotide identities; those below the diagonal and to the left show amino acid identities.

genetic lineage is widely distributed throughout east Asia.

The M segments of Solovey, Primorye, and AMR sequences formed a common phylogenetic lineage with high bootstrap support values, regardless of viral origin (Figure 2, A). Furthermore, H8205 shared a common lineage with Solovey and Primorye sequences. Another phylogenetic analysis, based on a different region of the M segment, showed that Chinese virus isolates (H8205, H3, H5, and B78) formed a distinct lineage within the Hantaan clade (Figure 2, B). The phylogenetic tree constructed for the S sequences (Figure 3) showed that Solovey sequences formed a single cluster, together with Maaji1 (a Korean isolate) and B78, in a common lineage with high bootstrap support values within the Hantaan clade.

To identify signature amino acids for each virus type, we compared the deduced partial amino acid sequences of their G2 regions using ClustalX multiple-sequence alignment (Figure 4). The presence of leucine or isoleucine at amino acid position (aa) 903 was unique to HTNV except for AMR lineage. The signature amino acids for SEOV were leucine at aa 918 and valine, isoleucine, and serine at aa 955-957. The sig-

Table 6. Comparison of nucleotide (bases 2737–2969)[a] and amino acid of M genome between those from Primorye patients, *Apodemus peninsulae*, and other hantaviruses

| | Nucleotide and amino acid identities %[b] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SL/AP61 | SL/AP63 | AMR/1169 | PRI/H1 | PRI/H2 | H8205 | AMR/4313 | HV114 | A9 | HTNV 76-118 | Hojo | FE | NC167 | DOB/Slo | SR-11 | PUUV |
| SL/AP61[c] | | **99.5** | **97.8** | **96.1** | **98.2** | **94.8** | **94.3** | 86.2 | 85.7 | 84.4 | 82.7 | 82.7 | 79.3 | 79.3 | 79.7 | 60.3 |
| SL/AP63 | **100** | | **97.8** | **92.2** | **94.3** | **94.8** | **94.3** | 85.7 | 85.3 | 84.0 | 82.3 | 83.1 | 78.8 | 80.1 | 81.4 | 60.7 |
| AMR/1169 | **94.8** | **94.8** | | **96.5** | **98.7** | **95.6** | **95.6** | 86.6 | 86.2 | 84.9 | 83.1 | 81.4 | 79.7 | 80.1 | 79.3 | 60.3 |
| PRI/H1 | **100** | **100** | **94.8** | | **96.9** | **93.5** | **92.2** | 84.0 | 83.6 | 83.1 | 82.3 | 80.6 | 79.3 | 78.8 | 79.3 | 60.3 |
| PRI/H2 | **98.7** | **98.7** | **93.5** | **98.7** | | **94.8** | **94.3** | 85.7 | 85.3 | 84.0 | 82.3 | 81.4 | 78.8 | 79.3 | 78.8 | 59.4 |
| H8205 | **100** | **100** | **94.8** | **100** | **98.7** | | **91.3** | 83.6 | 83.1 | 85.3 | 84.9 | 80.6 | 77.1 | 79.3 | 77.1 | 60.7 |
| AMR/4313 | **98.7** | **98.7** | **93.4** | **98.7** | **97.4** | **98.7** | | 85.7 | 85.3 | 83.6 | 81.8 | 82.7 | 78.0 | 78.0 | 78.8 | 59.9 |
| HV114 | 93.5 | 93.5 | 88.3 | 93.5 | 92.2 | 93.5 | 92.2 | | 99.5 | 86.6 | 84.4 | 87.9 | 78.4 | 75.8 | 83.1 | 51.9 |
| A9 | 93.5 | 93.5 | 88.3 | 93.5 | 92.2 | 93.5 | 92.2 | 98.9 | | 86.2 | 84.0 | 87.5 | 78.0 | 75.4 | 81.8 | 50.6 |
| HTNV76118 | 94.8 | 94.8 | 89.6 | 94.8 | 93.5 | 94.8 | 93.5 | 97.4 | 96.1 | | 94.6 | 88.7 | 79.7 | 78.4 | 76.7 | 59.9 |
| Hojo | 94.8 | 94.8 | 89.6 | 94.8 | 93.5 | 94.8 | 93.5 | 97.4 | 96.1 | 100 | | 87.9 | 78.0 | 78.8 | 76.7 | 51.5 |
| FE | 92.2 | 92.2 | 87.0 | 92.2 | 90.9 | 92.2 | 90.9 | 87.9 | 87.5 | 97.4 | 98.7 | | 75.8 | 73.7 | 78.4 | 59.9 |
| NC167 | 86.8 | 86.8 | 80.5 | 86.8 | 85.5 | 86.8 | 85.5 | 89.5 | 88.2 | 90.8 | 90.8 | 88.2 | | 75.4 | 77.5 | 49.3 |
| DOB/Slo | 88.3 | 88.3 | 83.1 | 88.3 | 87.0 | 88.3 | 87.0 | 88.3 | 87.0 | 87.0 | 87.0 | 84.4 | 81.6 | | 75.0 | 59.9 |
| SR11 | 83.1 | 83.1 | 79.2 | 83.1 | 81.8 | 83.1 | 81.8 | 83.1 | 81.8 | 81.8 | 81.8 | 83.1 | 80.3 | 80.5 | | 56.0 |
| PUUV | 53.2 | 53.2 | 53.2 | 53.2 | 51.9 | 53.2 | 51.9 | 62.9 | 62.5 | 51.9 | 61.6 | 53.2 | 61.6 | 49.4 | 61.2 | |

[a]Based on Haantan 76-118.
[b]Values above the diagonal and the right show nucleotide identities; those below the diagonal and to the left show amino acid identities.
[c]Values in bold show the close identities between those sequences. Abbreviations used: SL, Solovey; AMR, Amur; PRI, Primorye; HTNV, *Haantan virus*; FE, Far East virus; DOB, Dobrova; Slo, Slovenia; PUUV, *Puumala virus*

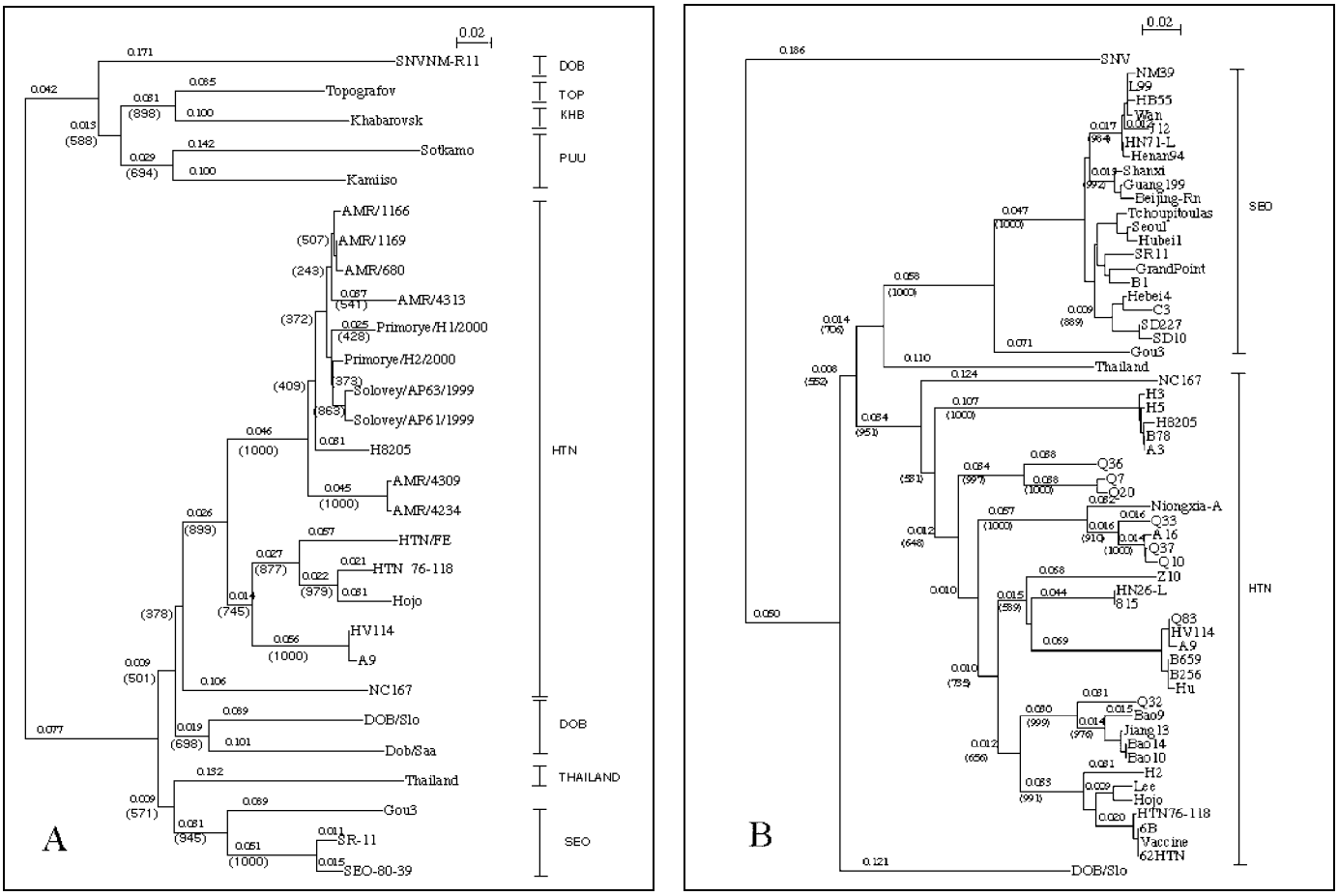


Figure 2. Phylogenetic trees of hantavirus (A) partial M (nt 2736–2968) and (B) partial M (nt 2001–2301) segments. Trees were constructed by using ClustalX (ver. 1.81) program. Numbers above the branches are distances and those in parentheses are bootstrap support values for 1000 replicates.

nature amino acids for AMR lineage were methionine at aa 932 and aspartic acid at aa 967.

## Discussion

Each hantavirus serotype or genotype is generally associated with a specific rodent host, and various rodent species act as reservoir animals and sources of human infection. Since contact between rodents and humans occurs frequently during agricultural and forestry activities, most infections have been reported in rural areas. However, an urban epidemic of HFRS caused by SEOV has also been reported (26). A large number of rodent species may serve as reservoir animals for pathogenic hantaviruses. For example, few researchers suspected that *P. maniculatus* could transmit highly virulent hantavirus to humans until SNV was identified (27,28). Later studies showed that the other viral agents of HPS such as NYV, BCCV, BAYV, and ANDV, were carried by *P. leucopus, S. hispidus* (29), *O. palustris* (30), and *O. longicaudatus* (31), respectively. We emphasize the importance of discovering the characteristics of hantaviruses found in endemic areas and identifying the primary hosts.

Although Far East Russia has long been considered an HFRS-endemic area, few reports describe the hantaviral sequences in this region, and information on reservoir animals carrying pathogenic hantaviruses is limited. Our studies therefore focused on determining the genetic characteristics of hantaviruses circulating in this geographic area. We identified *A. peninsulae* as the natural reservoir rodent for a hantavirus pathogenic for humans in Far East Russia. We also identified hantavirus sequences designated as Solovey and Primorye in *A. peninsulae* and HFRS patients, respectively; genetic analysis showed that these sequences were very closely related to each other. This information and the pathological findings from the HFRS case in which Primorye sequence was identified strongly suggest that the virus of Solovey sequence is the causative agent of HFRS. The nucleotide sequence and phylogenetic analysis also showed that Solovey and Primorye sequences were most closely related to AMR and H8205 sequences from patients in Russia and China, but were clearly distinguishable from the prototype of Hantaan virus. Genetic and phylogenetic analysis indicated that Solovey and Primorye sequences were closely related to AMR, Maaji1, H8205, and B78 sequences, viruses derived from distant areas. While Solovey sequences were identified in a suburb of Vladivostok and PRI sequences in two villages 400 km and 600 km from Vladivostok, the H8205 and B78 viruses were derived in

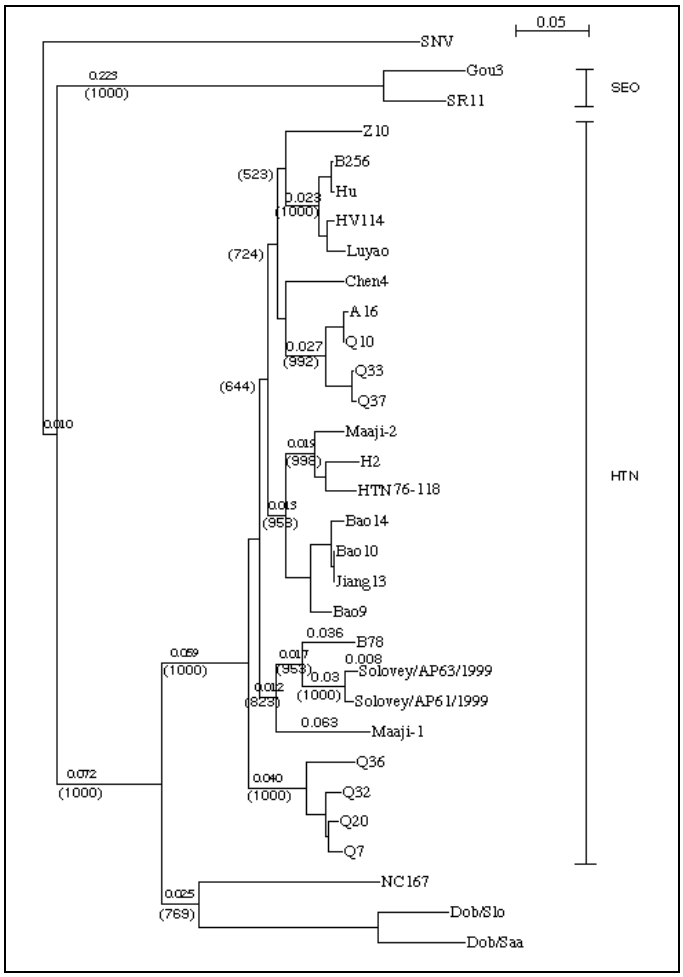


Figure 3. Phylogenetic tree of hantavirus partial S (nt 1216-1666) segments. Tree was constructed by using ClustalX (ver. 1.81) program. Numbers above the branches are distances and in parentheses are bootstrap support values for 1000 replicates.

China, and Maaji1 was isolated in Korea. *A. peninsulae* is distributed in the same region where *A. agrarius* is prevalent in Korea (PW Lee, pers. comm.). Recently, AMR sequences were found in both HFRS patients and *A. peninsulae* (11). We suggest that some of the viruses circulating in the area of this study cause severe HFRS and are carried by the same host species, *A. peninsulae*. Comparison of the deduced hantaviral amino acid sequences showed that aspartic acid and methionine represented signature amino acids for AMR genetic lineage, regardless of the region in which the virus was identified or its origin (Figure 4). These signature amino acids may be used to distinguish AMR genetic lineage from other hantaviruses. We conclude from our results that *A. peninsulae* carries a hantavirus that is pathogenic for humans. Since *A. peninsulae* is widely distributed in Far East Russia, China, Korea, and Japan, this hantavirus and associated cases of HFRS may also be widely distributed.

In the kidney tissue of one HFRS patient (no. 2) from Primorye region, we detected pathologic changes typical of severe HFRS caused by hantavirus infection (32–35). We also

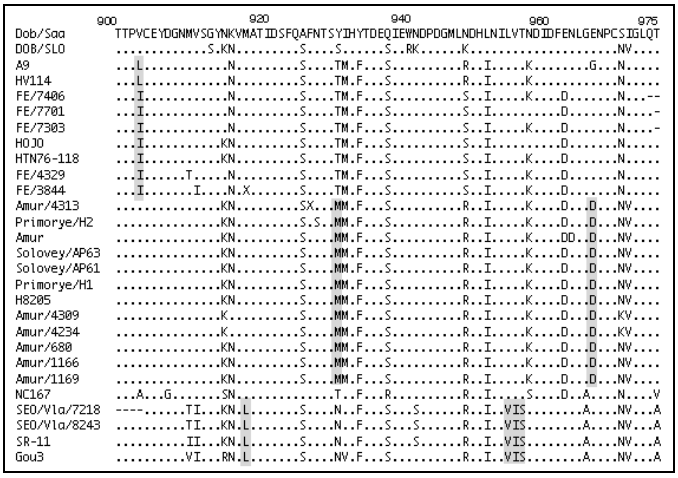


Figure 4. Multiple alignment of partial deduced amino acid sequences of G2 region of hantaviruses. Amino acid sequences analyzed by using ClustalX (ver. 1.8) program. Amino acid positions indicated above sequences based on Haantan 76-118. First line shows the deduced amino acid of Dobrova/Saarema. Dots represent amino acids that are identical to those at corresponding positions in Dobrova/Saarema sequence. Amino acids that differ from those in the sequence are indicated at relevant positions. Hyphens are used in areas where amino acid sequence is not available. Signature amino acids are shaded.

detected and sequenced the partial M segment in the spleen of the same patient. However, we could not detect the viral antigen in the kidney samples, possibly because of low levels of the virus in the kidneys of this patient. Nested PCR allowed the amplification of viral M segments from the spleen, but not from kidney, of this patient.

Through epizootiologic, clinical, pathologic, and sequencing studies, we identified a hantavirus carried by *A. peninsulae* as one of the causative agents of HFRS. We think that this information may be helpful in preventing human infections in East Asia. Controversy persists over whether *A. peninsulae* carries a distinct virus type or a subtype of HTNV. A similar question arises with Dobrava/Slovenia and Dobrava/Saaremaa, which are carried by *A. flavicollis* and *A. agrarius*, respectively. The S segment identities between Dobrava/Slovenia and Dobrava/Saaremaa (both obtained from GenBank for comparison purposes) were 87.8% (nucleotide) and 92.7% (amino acid). Similarly, the nucleotide and amino acid sequence identities of the S segments of Solovey sequences and HTN 76-118 were 82.7% and 92.2%, respectively. We suggest that Solovey sequences belong to a sublineage within the HTNV clade.

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# West Nile Virus Outbreak in Horses, Southern France, 2000: Results of a Serosurvey

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During late summer and autumn 2000, a West Nile fever outbreak in southern France resulted in 76 equine clinical cases; 21 horses died. We report the results of a large serosurvey of all equines within a 10-km radius of laboratory-confirmed cases. Blood samples were obtained from 5,107 equines, distributed in groups of 1 to 91 animals. West Nile virus immunoglobulin (Ig) G antibodies were found in 8.5% of animals (n=432). Forty-two percent of the IgG-positive animals were also IgM positive. Horses living in small groups were more affected than those in large groups. The results suggest that West Nile virus is not endemic in the affected area, the Camargue; rather, sporadic outbreaks are separated by long silent periods.

*West Nile virus* (WNV) is an arbovirus of the genus *Flavivirus*, family *Flaviridae*. Its natural transmission cycle involves birds and mosquitoes, mainly of the *Culex* genus. During late summer and autumn 2000, a WNV outbreak in southern France resulted in 76 clinical cases in equines; 21 horses died (1). The cases occurred near the Camargue region, a large wet area that corresponds to the delta of the Rhône River (Figure 1), near the Mediterranean coast. The area has a rich avifauna (2,3); >300 bird species, mostly water birds have been observed there. Among these species, some are migratory: Camargue is an important resting area for birds migrating between western Africa and northern Europe. Camargue is also a breeding area for some species and a wintering area for others. Mosquito density is high in this wet area (3,4). Among *Culex* species, *C. pipiens* and *C. modestus* are the most abundant.

In France, the first reported outbreak occurred in humans and equines during the summer of 1962 in the south of the country (5,6). Equine cases occurred both in Camargue (approximately 30 cases) and in a neighboring dry area (Figure 1; approximately 50 cases). From 1963 through 1964, a serosurvey was conducted in both areas: 6 of 37 horses were found positive for WNV (6).

The 2000 outbreak occurred west of Camargue (Figure 1), where the landscape features two very different biotopes. The coast is mainly wet areas with rice fields, numerous ponds, and marshes. North of these wet areas are dry areas with vineyards, farming areas, and typical Mediterranean vegetation. Most of the cases occurred in the dry areas (Figure 1).

On September 6, 2000, positive serologic results (immunoglobulin [Ig] G and IgM) were first found in two horse samples. Two days later, WNV infection was confirmed by detection of viral RNA in a brain biopsy (1). Clinical cases were observed until November 2. No abnormal deaths were observed in birds, and a serosurvey was conducted in November and December 2000 with captive ducks and wild birds (sparrows, gulls, and magpies). Positive results were found in one gull, eight ducks, and four magpies (7). Mosquitoes were also collected in the outbreak area, but none of the pools was found positive. No human cases were reported; however, WNV neutralizing antibodies were detected in three gamekeepers working in the area, one of whom also had IgM antibodies (1).

Experimental studies and sequential samples collected from naturally infected horses have shown that IgM antibodies become detectable 8–10 days post-infection and persist <2–3 months (8,9). WNV neutralizing antibodies can persist >2 years after infection (9). No published data could be found about the evolution of the WNV IgG response in horses; however, IgG neutralizing antibodies may persist several years after infection.

After the first horse case was confirmed, a serosurvey was ordered by the animal health authorities on all equines located

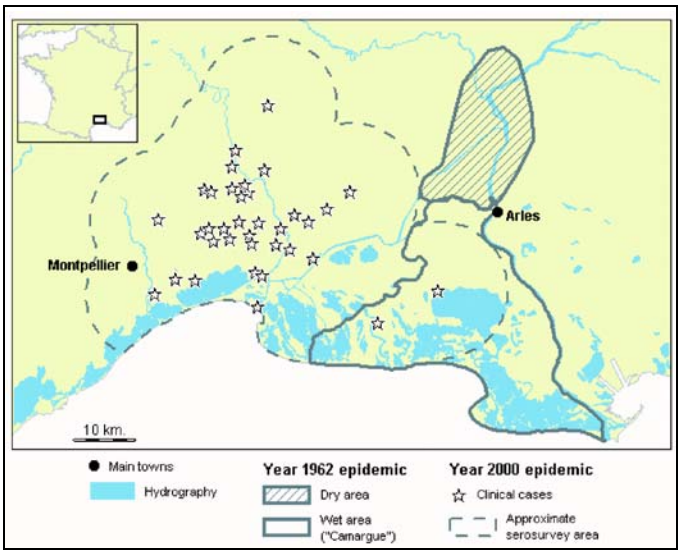


Figure 1. West Nile virus clinical infection in equines in southern France, 1962–2000.

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within a 10-km radius of laboratory-confirmed clinical cases (1). Preventive measures included prohibiting movements of horses inside this perimeter. We report the results of this serosurvey, the first large-scale serosurvey conducted in equines worldwide.

## Material and Methods

Blood samples were taken from all the equines within 10 km of the laboratory-confirmed cases (Figure 1). The use of a 10-km radius area for control measures is common in animal diseases control plans (e.g., against foot and mouth disease or classical swine fever). The sera were processed and tested for WNV IgG and IgM antibodies as described (1). Animals were first tested for WNV IgG antibodies, and because of logistic constraints, only positive sera were then tested for IgM antibodies. A positive animal was defined as an IgG-positive animal. A positive group was defined as a group in which at least one animal was IgG positive.

For each animal, a form was completed by a veterinarian. Along with other data (date, names and addresses of the veterinarian and the animal caretaker), this form noted the species and breed of the animal, its age and sex, location, and the size of the group in which the animal was included on the day the sample was taken (i.e., the number of equines at the same place).

The individual serologic results and the data on the forms were collected in a Microsoft Access database (Microsoft Corp., Redmond, WA). The horses were grouped by sex (geldings, mares, and stallions) and age (<1 year, 1–2 years, 3–5 years, 6–10 years, 11–15 years, 16–20 years, 21–25 years, and >25 years). Breeds were grouped into four classes according to their typical management conditions: the pure breeds (expensive animals kept in stalls at night and pastures by day), the Camargue breed (an ancient breed of rugged saddle horses or half-wild animals originating in the survey area, always kept outside), pony breeds (often living in riding stables and used every day for short rides) and other breeds (donkeys and other equines, typically kept outside in small groups on pastures). Four frequency categories (all having the same number of animals) were defined for group sizes: 1–2 animals, 3–6 animals, 7–25 animals, and >25 animals.

Maps were produced with MapInfo software (MapInfo Corp., Troy, NY). The geographic data describing animal locations was the name of the "commune," the smallest administrative French subdivision, which corresponds to an English parish.

Statistical analyses were done with SAS software (SAS Institute Inc., Cary, NC). A bivariate analysis was first performed, crossing each variable (age, sex, breed, and group size) with the prevalence. The chi square test was calculated for the four variables. Prevalence ratios were computed for breed and group size, using the category with the lowest prevalence as a reference. For animals groups, chi square tests and prevalence ratios were computed both at the animal level (crossing the group size class with the animal-level prevalence) and the group level (crossing the group size class with the group-level prevalence). Finally, a logistic regression was conducted. Animals for which the age, sex, breed, or group size had not been indicated were excluded from the data set. The dependent variable of the model was the serologic status of animals, and the independent variables were their age class, sex, breed, and group size. (The location of animals could not be included in the model because many regression classes would have been empty.) For each variable, the reference class was the category with the lowest prevalence.

## Results

### Descriptive Analyses

Fifty veterinarians took blood samples from 5,107 equines (4,776 horses and 91 donkeys) distributed in 1,429 groups. The 14-week serosurvey began on September 16 (week 38) and ended December 15. The survey area was approximately 2,500 $km^2$ and covered (partially or totally) 99 communes.

The age of 4,749 animals ranged from birth to 40 years. Half the animals were geldings (48%, n=2,345), 32% were mares (n=1,533), and 20% were stallions (n=951). Of 53 breeds noted for 4,867 animals, the predominant breed was the Camargue (36%; n=1,743). Group sizes ranged from 1 to 91 animals, but most of the groups (70%; n=1,006) were small (1–2 animals). The mean group size was 3.6 animals.

Of the 5,107 animals, 432 were IgG positive (8.5%; 95% confidence interval [CI] 7.7 to 9.2). Almost half (n=182) were also IgM positive (42.1%; 95% CI 40.8 to 43.5). The group-level seroprevalence of the positive groups was higher than the animal-level seroprevalence: 19.2% (n=274) (95% CI 17.1 to 21.2).

### Time Distribution

More than 50% of the samples were taken during the first 3 weeks; 90% of the samples had been taken at the end of week 6 of the study (Figure 2). IgG-positive animals were identified throughout the 14-week study period: the last positive animals were found during week 12. Animals that were both IgG- and

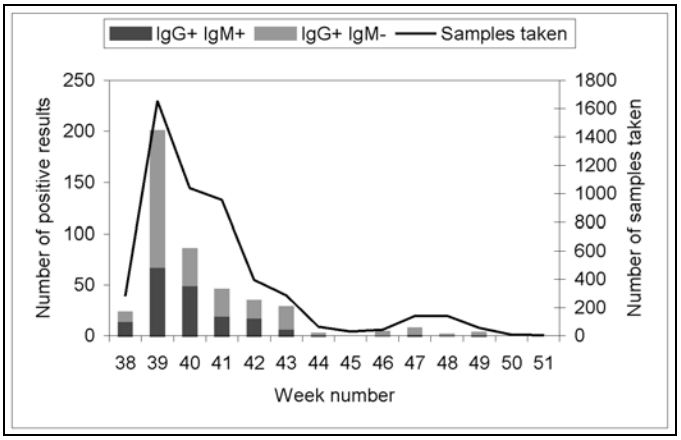


Figure 2. West Nile virus serosurvey, Camargue, France, 2000: number of samples and seropositive animals, by week.

IgM-positive were also found until week 12, with a slight decrease over time (15 of 23 during week 1 and 1 of 3 during week 12).

### Geographic Distribution

The map of the number of serum samples per commune shows that the geographic distribution of horses is not homogeneous (Figure 3): fewer samples were taken from horses in the north of the survey area, where land is more devoted to vineyards and agriculture, than in the south, in or near wet areas. The overall animal density is approximately 2 animals per km2. However, the density in some communes is >16 animals per km2.

The prevalence by commune (Figure 4) is higher in the center of the area, as is also the case for the geographic distribution of clinical cases (Figure 1). The main part of this "hot spot" is not in a wet area, but in a rather dry area. In this focal point, the prevalence exceeds 30% in eight communes and reaches 58% in one (28 positive of 48 animals). A similar pattern is found for the group-level seroprevalence, with higher rates, exceeding 50% in nine communes and reaching 88% in one, with 21 positive groups of 24.

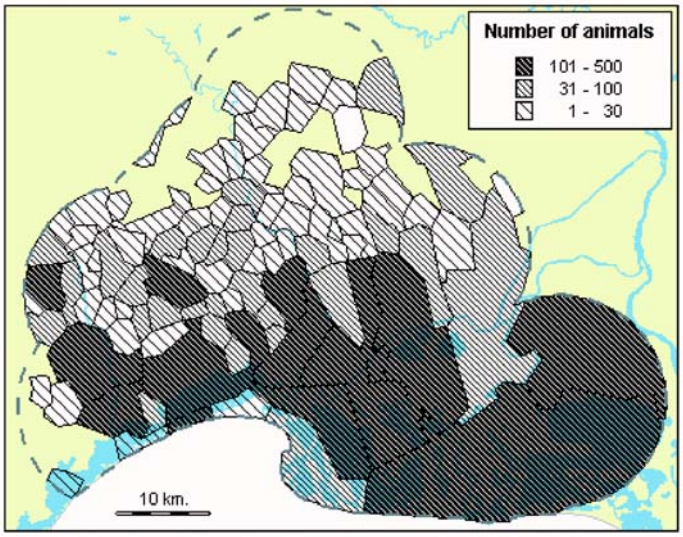


Figure 3. West Nile virus serosurvey, Camargue, France, 2000: geographic distribution of the animals sampled, by commune (n=5,905).

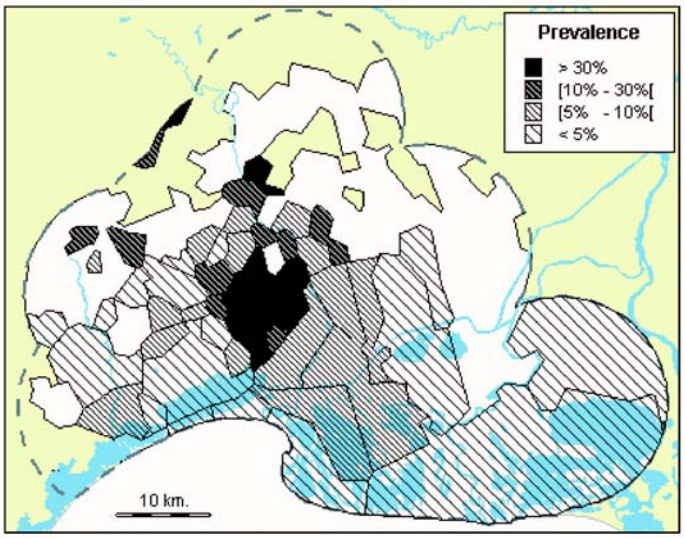


Figure 4. Immunoglobulin G anti-West Nile virus prevalence in equines, by commune, Camargue, France, 2000 (n=5,095).

### Bivariate Analysis

No significant difference was found in the serologic status of animals according to their distribution by age (chi square 6.63; p=0.47; Table 1) and by sex (chi square 2.65; p=0.27). A significant difference was found in the serologic status of animals by breed (chi square=11.12; p=0.01; Table 2), with a prevalence ratio of 1.5 (95% CI 1.2 to 1.9) for the Camargue breed. A significant difference was also found in the serologic status of animals by group size (chi square 54.11; p<0.0001; Table 3), and decreasing prevalence ratio was observed as group size increased. At the group level, a significant difference was also found in the serologic status of groups according to their distribution by size (chi square 55.68; p<0.0001; Table 4); however, increasing prevalence ratios were observed with increasing group size.

### Logistic Regression Results

The data set used for the logistic regression (animals for which the age, sex, breed, and group size were known) contained 4,597 records. The reference classes were as follows: <1 year, mares, other breeds, and >25 animals. A slight effect was found for the Camargue breed (odds ratio [OR] 1.40; 95% CI 1.08 to 1.82). Conversely, the main effect was attributed to the group size variable, with decreasing ORs with increasing group size: 2.18 for groups of 1–2 animals (95% CI 1.60 to 2.96) and 1.81 for groups of 3–6 animals (Table 5).

Belonging to a small group and, to a lesser extent, being of the Camargue breed appeared to be risk factors for seropositivity. Comparison of the eight most affected communes with the rest of the survey area showed no significant differences by age, sex, breed, and group size (data not shown). Therefore, these risk factors are not explained by an overrepresentation of Camargue horses and of small groups near the epidemic hot spot.

Table 1. Immunoglobulin G anti-West Nile virus prevalence in equines by age group, Camargue, France, 2000

| Age (yrs) | Total | Positive (%) |
|---|---|---|
| <1 | 33 | 2 (6.1) |
| 1–2 | 368 | 44 (12.0) |
| 3–5 | 938 | 88 (9.4) |
| 6–10 | 1,559 | 124 (8.0) |
| 11–15 | 975 | 87 (8.9) |
| 16–20 | 555 | 50 (9.0) |
| 21–25 | 229 | 21 (9.2) |
| >25 | 92 | 9 (9.8) |
| Total | 4,749 | 425 (8.9) |

[a]Chi square = 6.63 (7 degrees of freedom); p=0.47.

Table 2. Immunoglobulin G anti-West Nile virus prevalence in equines by breed, Camargue, France, 2000[a]

| Breed class | Total | Positive (%) | Prevalence ratio | 95% CI |
|---|---|---|---|---|
| Pure breeds | 1,252 | 108 (8.6) | 1.2 | 0.9 to 1.7 |
| Camargue | 1,743 | 183 (10.5) | 1.5 | 1.2 to1.9 |
| Ponies | 497 | 38 (7.6) | 1.0 | 0.7 to1.5 |
| Other | 1,375 | 100 (7.3) | Ref. | |
| Total | 4,867 | 429 (8.8) | | |

[a]Chi square=11.12 (3 degrees of freedom), p=0.01; 95% CI = 95% confidence intervals.

## Discussion

### Serosurvey Results

The geographic variations of the seroprevalence show that the epidemic hot spot was not located in a wet area, but several km to the north, in a rather dry area, even though the horse density was roughly the same in both areas. Moreover, antibodies were found in 8% of the captive mallards at a large pond in the south of the perimeter, and in four magpies of 18 captured a few km to the north, near the horse epidemic hot spot (7). The existence and the specific location of the epidemic hot spot could therefore be explained by primary circulation of the virus in water birds in wet areas, followed by an amplification of this circulation by synanthropic bird species, involving spread from wet areas to dry areas, where these birds usually live.

Data analysis showed no age effect. Several serosurveys in human populations have shown that, when WNV circulation is endemic in a given area, the seroprevalence tends to increase with age (10–13). A similar result was found in Egyptian equines in 1963 (14). However, no such increase was found in 1999 in dogs in New York City, where the virus was thought to have been absent before that year (15). Assuming that anti-WNV IgG usually persists several years after infection and that most of the animals had lived in the survey area for a long time (and that all age classes are roughly equally exposed to infecting bites), these results suggest the absence of an endemic circulation of the WNV in the area. However, the existence of an endemic transmission cycle, geographically restricted to marshes (where half-wild horses live that could not be sampled) cannot be excluded.

A breed effect was observed in that the prevalence was higher in Camargue horses. This result reflects the usual living conditions of these rugged horses, always living outside and therefore more likely to be exposed to infectious bites.

Group size had two opposite effects on seroprevalence, depending on the unit used for calculation. At the group level, the increase in seroprevalence with increasing group size is a classical finding in veterinary epidemiology: assuming all animals in a given group are exposed to the same low-level probability of infection, the more animals in the group, the higher the probability that one (or more) of them become infected. At the individual level, the decrease of the seroprevalence rate with increasing group size may be the result of two factors. First, the sizes of the groups reflect different uses and management conditions of the horses: animals kept in large groups may benefit from better management practices (e.g., stabling at night, which could reduce their exposure to infecting bites). However, this result could also reflect a low spatial density of infectious vectors: assuming a vector does not bite all animals of a given group but only a few of them, large groups would have a protective effect. The high density of horses in the area could also help explain the absence of reported human cases as a result of a possible zooprophylactic effect of domestic animals, as pointed out by Komar et al. (15) (domestic animals may divert infectious bites from human hosts). Because of lack of data about the primary use and the management conditions of the horses, we could not evaluate the respective importance of these two factors on the protective effect of large groups.

### Limits and Biases

The survey was intended to be comprehensive for all equines located <10 km from laboratory-confirmed clinical cases. Movements of horses in and out of the area probably occurred at the beginning of the outbreak, before the restrictive measures taken by the animal health authorities were in place. Some of the Camargue horses are half-wild and live year-round in marshes; for practical reasons, these half-wild animals were not sampled. However, even if the survey was not strictly exhaustive, the 5,107-equine sample is certainly highly representative of the domestic equines in the area.

Having tested only the IgG-positive sera for IgM antibodies is an obvious bias: recently infected animals (with IgM antibodies but without IgG antibodies) that did not show clinical signs would have been missed. The seroprevalence figures obtained may be underestimated for domestic equines. Belonging to the Camargue breed was identified as a seropositivity risk factor and half-wild animals that were not sampled belong to this breed; therefore, the seroprevalence rate is probably also underestimated for the whole equine population of the area (domestic and half-wild equines).

The geographic distribution of the positive results shows that, in the east part of the survey area (and to a lesser extent the west), some positive results were still found near the area

Table 3. Immunoglobulin G anti-West Nile virus individual-level prevalence in equines by group size, Camargue, France, 2000[a]

| Group size | Total | Positive (%) | Prevalence ratio | 95% CI |
|---|---|---|---|---|
| 1–2 animals | 1,353 | 165 (12.2) | 2.2 | 1.6 to 3.0 |
| 3–6 animals | 1,118 | 114 (10.2) | 1.8 | 1.3 to 2.5 |
| 7–25 animals | 1,355 | 87 (6.4) | 1.1 | 0.8 to 1.5 |
| >25 animals | 1,281 | 55 (5.1) | Ref. | |
| Total | 5,107 | 432 (9.7) | | |

[a] Chi square = 54.11 (3 degrees of freedom), p<0.0001; 95% CI = 95% confidence intervals.

Table 4. Immunoglobulin G anti-West Nile virus group level prevalence in equine groups by group size, Camargue, France, 2000

| Size class | Total | Positive (%) | Prevalence ratio | 95% CI |
|---|---|---|---|---|
| 1–2 | 1,006 | 149 (14.8%) | Ref. | |
| 3–6 | 294 | 73 (24.8%) | 1.9 | 1.4-2.6 |
| 7–25 | 98 | 40 (40.8%) | 4.0 | 2.6-6.1 |
| >25 | 31 | 12 (38.7%) | 3.6 | 1.7-7.6 |
| Total | 1,429 | 274 (19.2%) | | |

[a] Chi square = 55.68 (3 degrees of freedom), p<0.0001; 95% CI, 95% confidence intervals.

boundary: more positive results may have been included if the perimeter had been larger.

The serosurvey was carried out over a 14-week period; most of the samples were taken during the first 6 weeks. Because recent WNV circulation was detected (through IgM-positive results) until week 12, some animals tested at the beginning of the study were probably infected later, and the prevalence might have been higher if the study had been conducted during winter.

### Comparison with Other Studies

Few serosurveys have been conducted in equines. Survey results can be usefully compared between each other in disease-endemic areas (14); however, comparison is difficult if the survey is in epidemic areas. In such areas, the seroprevalence rate depends on the definition of the survey perimeter (which varies between studies). For example, in our study, if a radius of 15 km around cases had been used to define the survey perimeter instead of 10 km, the seroprevalence rate probably would have been lower.

In an endemic area, Egypt in 1959 (14), high seroprevalence rates were found, with figures ranging from 36% to 81%. Conversely, in a newly infected area, New York City in 1999 (15), a much lower rate was found: 2.7% of 71 horses (95% CI 0.3 to 9.5). The seroprevalence we observed is closer to the latter rate. However, in the Egyptian survey, the 15% seroprevalence rate in young horses (≤2 yrs) is closer to our result: this figure better reflects the yearly infection rate and thus the infection pressure.

The seroprevalence rate can also be compared with the results of two earlier serosurveys conducted in the studied area. After the 1962 epidemic (Figure 1), in 37 sera collected in 1963–1964 from horses that did not have clinical signs the year before, 6 were positive (16.2%; 95% CI 4.3 to 28.8) (6). From 1975 to 1979, a low frequency of antibody response against WNV was observed in 99 horse samples (2%) (16). Therefore, in the studied region, WNV epidemics may occur sporadically and be followed by long silent periods.

The results of this study, the first large-scale WNV serosurvey conducted in equines, show that seroprevalence rate does not increase with age. Assuming that, as neutralizing antibodies, IgG antibodies persist several years after infection,

Table 5. Immunoglobulin G anti-West Nile virus prevalence in equines, by age, sex, breed, and group size, Camargue, France, 2000

| Variable | OR estimate[a] | 95% CI |
|---|---|---|
| **Age (years)** | | |
| <1 | Ref | |
| 1–2 | 1.83 | 0.42 to7.99 |
| 3–5 | 1.39 | 0.32 to 5.98 |
| 6–10 | 1.13 | 0.26 to 4.86 |
| 11–15 | 1.25 | 0.29 to 5.41 |
| 16–20 | 1.24 | 0.28 to 5.43 |
| 21–25 | 1.20 | 0.26 to 5.48 |
| >25 | 1.24 | 0.25 to 6.15 |
| **Sex** | | |
| Mare | Ref. | |
| Gelding | 1.13 | 0.89 to 1.45 |
| Stallion | 1.18 | 0.88 to 1.58 |
| **Breed** | | |
| Pure breeds | 1.30 | 0.97 to 1.74 |
| Camargue | 1.40 | 1.08 to 1.82 |
| Ponies | 1.15 | 0.77 to 1.72 |
| Other | Ref. | |
| **Group size** | | |
| 1–2 | 2.18 | 1.60 to 2.96 |
| 3–6 | 1.81 | 1.31 to 2.52 |
| 7–25 | 1.09 | 0.77 to 1.53 |
| >25 | Ref. | |

[a]OR, odds ratio; 95% CI, 95% confidence intervals.

this result (and the WNV history in the studied area since 1962) suggest that the Camargue is an epidemic area, with outbreaks that occur periodically and are separated by long silent periods. The seroprevalence level, especially for animal groups, indicates that virus circulation was intense during the 2000 epidemic. This intense virus circulation and the location of the epidemic focus in a dry area could be explained by the amplification by synanthropic bird species in dry areas, from a primary circulation in wet areas in water birds. The survey results do not allow us to assert whether this primary circulation is permanent or not (the virus being periodically reintroduced by migratory birds). However, the survey results suggest that, if the primary cycle is permanent, it is restricted to small marshy areas. Two further studies, a serologic follow-up of captive ducks and a serosurvey focused on horses living in the marshy areas, will be conducted in 2001–2002; these studies should allow us to refine the epidemiologic status of this primary cycle.

**Acknowledgments**

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Dr. Durand is a researcher at the Agence Française de Sécurité Sanitaire des Aliments. His main research topics are the epidemiology and the modeling of the major animal diseases (foot-and-mouth disease, classical swine fever) and of emerging or reemerging diseases (West Nile fever, scrapie).

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# Outbreak of Cyclosporiasis Associated with Imported Raspberries, Philadelphia, Pennsylvania, 2000

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An outbreak of cyclosporiasis occurred in attendees of a wedding reception held in Philadelphia, Pennsylvania, on June 10, 2000. In a retrospective cohort study, 54 (68.4%) of the 79 interviewed guests and members of the wedding party met the case definition. The wedding cake, which had a cream filling that included raspberries, was the food item most strongly associated with illness (multivariate relative risk, 5.9; 95% confidence interval, 3.6 to 10.5). Leftover cake was positive for *Cyclospora* DNA by polymerase chain reaction analyses. Sequencing of the amplified fragments confirmed that the organism was *Cyclospora cayetanensis*. The year 2000 was the fifth year since 1995 that outbreaks of cyclosporiasis definitely or probably associated with Guatemalan raspberries have occurred in the spring in North America. Additionally, this is the second documented U.S. outbreak, and the first associated with raspberries, for which *Cyclospora* has been detected in the epidemiologically implicated food item.

On June 10, 2000, a total of 83 persons attended a catered wedding reception in Pennsylvania. Approximately 8 days later, the bride notified the local health department that she, her husband, and many guests at the reception had a gastrointestinal illness. Stool specimens from attendees were positive for oocysts of the coccidian parasite *Cyclospora cayetanensis*, and an epidemiologic investigation was begun by the Philadelphia Department of Public Health and the Centers for Disease Control and Prevention (CDC).

Previous foodborne outbreaks of cyclosporiasis in the United States have been associated with consumption of fresh raspberries, mesclun lettuce, and basil (1–8). An important feature of the biology of *Cyclospora* is that oocysts excreted in feces require days to weeks outside the host to sporulate and thus to become infectious. However, the minimum time required for sporulation is unknown. *Cyclospora* oocysts must survive in the environment long enough to sporulate and infect a susceptible host, which suggests that they are very hardy.

## Methods

### Epidemiologic Investigation

A retrospective cohort study was conducted by administering a structured questionnaire by telephone about symptoms and event-related exposures. A case of cyclosporiasis was defined as onset of illness 1–14 days after the reception and either (a) one positive stool specimen and at least one gastrointestinal symptom (i.e., loose stools, nausea, vomiting, stomach cramps, gas/bloating, loss of appetite, and unintentional weight loss) or constitutional symptom (i.e., fever, chills, muscle aches, generalized body aches, and headaches); (b) any loose stools in a 24-hour period and at least one other gastrointestinal or constitutional symptom; or (c) a total of at least three gastrointestinal symptoms.

We attempted to identify more cases of cyclosporiasis by having the Pennsylvania State Bureau of Epidemiology alert health departments throughout the state of the investigation. We also requested the catering company to contact the six groups to which they had served raspberries during the period June 2–4 that might have been from the suspected raspberry shipments.

### Statistical Analyses

Univariate relative risks and 95% confidence intervals (CIs) were calculated by using Epi-Info, version 6.04 (CDC). Multivariate Poisson regression analyses were conducted by using SAS, version 8.0 for Windows (SAS Institute Inc., Cary, NC).

### Laboratory Investigation

The local diagnostic reference laboratory examined stool specimens from five patients for *Cyclospora* oocysts by Kinyoun's modified acid-fast technique. CDC reevaluated specimens from three of these patients either by examining slides made by the local laboratory or by UV fluorescence microscopy of a stool specimen concentrated by the formalin-ethyl acetate technique. An unknown number of specimens were tested at the local diagnostic reference laboratory for *Cryptosporidium parvum*; *Salmonella*, *Shigella*, and *Campylobacter* spp.; and *Escherichia coli* O157:H7.

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CDC and the Food and Drug Administration (FDA) used molecular methods to examine leftover wedding cake, which the bride and groom had frozen, for *Cyclospora*. FDA's methods were as follows. One-gram portions of the icing from the top of the wedding cake, the raspberry filling, and the cake itself were processed for molecular analyses by using 20 mL of Tris buffer (containing 100 mM Tris-HCL, pH 8.0, 10 mM EDTA, 150 mM NaCl) and 5 mL of ethyl acetate. The samples were shaken vigorously, heated at 56°C, and gently agitated for 30 min. The samples were centrifuged for 15 min at 4500 x *g* at 15°C. The organic phase, the interface, and the aqueous (upper) phase were aspirated. Next, the pellet was washed twice with 25 mL of Tris buffer and centrifuged as above. The pellet was suspended in 4 mL of Tris buffer, layered over a 4-mL Optiprep solution (Invitrogen Corporation, Carlsbad, CA), and diluted 1:1 with the Tris buffer. The samples were centrifuged for 15 min at 250 x *g* at 15°C. The aqueous phase, the interface, and 1.5 mL of the upper portion of the Optiprep layer were removed and saved in a 15-mL centrifuge tube. The remaining pellet and Optiprep solution were discarded. All saved samples were diluted further with 2–3 mL of Tris buffer and centrifuged for 10 min at 20,000 x *g*.

The supernatant from each sample was aspirated to within 1 mL of the bottom, and the pellets were suspended in this volume and transferred to a 1.5-mL microcentrifuge tube. The samples were then centrifuged for 2 min in a microcentrifuge at 14,000 x *g,* and the supernatant was aspirated. The final pellets were suspended in 50 µL of distilled water, and a 10-µL volume of each sample was spotted onto Flinders Technologies of Australia (FTA) filters (Fitzco, Inc., Maple Plain, MN) for DNA extraction and subsequent polymerase chain reaction (PCR) analyses as described previously (9).

CDC's methods were as follows. DNA was extracted directly from nine randomly selected 1-g portions of raspberry filling from the cake by using selected components from the FastDNA kit (BIO 101, Inc., Vista, CA). The portions were placed into tubes containing disruption beads that were included in the kit, and DNA extraction was performed as described (10).

Nested PCR amplification was performed by using primers modified from those described elsewhere (11). The restriction sites *Eco*RI and *Bam*HI were removed from the 5′ end, and bases matching the *C. cayetanensis* small subunit (SSU) rRNA coding region were added. Thus, primers CYCF1 (5′-ATTACCCAATGAAAACAGTTT-3′) and CYCR2 (5′-TGCAGGAGAAGCCAAGGTAGG-3′) were used for the first step of nested amplification; the preamplified fragment was nested with primers CYCF3 (5′-GCCTTCCGCGCTTCGCTGCGT-3′) and CYCR4 (5′- TCGTCTTCAAACCCCCTACTG-3′). Amplification with these primers generates a 294-bp fragment from the *C. cayetanensis* SSUrRNA coding region.

Besides the nested PCR, a 636-bp fragment from the *Cyclospora* sp. SSUrRNA, flanked by primers CYCF1/CYCR2, was amplified and sequenced to identify the parasite at the species level. This fragment contains substitutions that allow differentiation among the *Cyclospora* spp. for which the sequence of the SSUrRNA coding region is available in GenBank (i.e., *C. cayetanensis*, *C. cercopitheci*, *C. colobi*, and *C. papionis*). Sequencing reactions were performed on PCR products that had been purified with the StrataPrep PCR Purification Kit (Stratagene, La Jolla, CA). Sequencing reactions, analyses, and assemblage of sequences were performed as described (12).

### Environmental and Source Investigations

The reception facility was inspected, and food-handling and storage practices were observed. Information about illness in the catering staff was collected by examining the caterer's sick logs. The caterer was also asked to contact the health department if staff called in sick. Ill staff members would then be asked whether they had had a gastrointestinal illness during the 3 weeks before and after the reception.

The caterer and the distributors that supplied his company were interviewed, and shipping documents were obtained to determine the source of the raspberries used in the cake (13). In cooperation with the Guatemalan government, FDA inspected one of the farms that could have supplied the raspberries served at the wedding; this farm also could have supplied the raspberries for an event in another state in 2000 that was associated with an outbreak of cyclosporiasis.

## Results

### Epidemiologic Investigation

Seventy-nine (95.2%) of the 83 attendees of the wedding reception were interviewed. Fifty-four (68.4%) of the 79 had illness that met the case definition. Five (9.3%) of the 54 case-patients had laboratory-confirmed cyclosporiasis, and 49 (90.7%) of the 54 case-patients had clinically defined cyclosporiasis. All case-patients who met part (c) of the case definition also met part (b). Therefore, parts (b) and (c) were combined to represent the clinically defined category of the case definition. The median incubation period was 7 days (range 1–9 days) (Figure 1). The symptoms associated with illness are listed in Table 1.

Several food items were significantly associated with illness in univariate analyses (Table 2). However, only the cake, which had a cream filling that included pieces of raspberries, was significantly associated with illness in multivariate analyses (relative risk 5.9; 95% CI 3.6 to 10.5). Mesclun lettuce was used as a garnish for several food items, and fresh basil was served in one food item; neither was significantly associated with illness.

We did not identify additional cases through the Pennsylvania State Bureau of Epidemiology's alert to local health departments. Moreover, the catering company contacted the six groups to which they served raspberries during June 2–4, and no additional cases were identified.

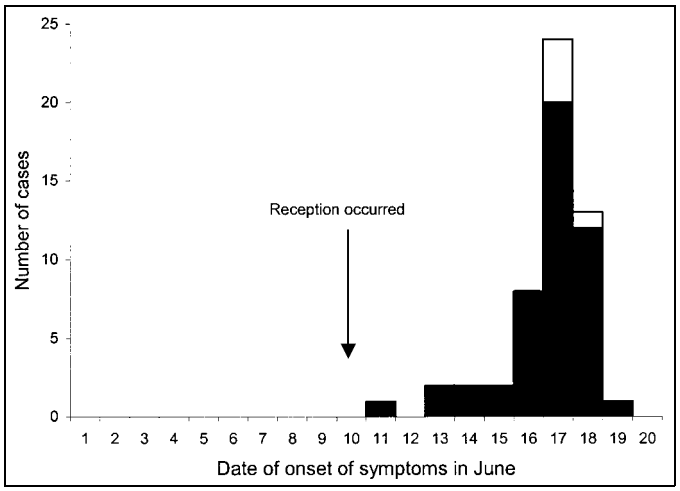


Figure 1. Dates of onset of symptoms in laboratory-confirmed case-patients (white box; n = 5) and clinically defined case-patients (black box; n = 49) who attended the wedding reception in June 2000 in Pennsylvania. The exact date of onset of symptoms was not specified for one case-patient who became ill after the reception.

### Laboratory Investigation

For three of the five case-patients who had laboratory-confirmed cyclosporiasis (Table 1), stool specimens were sent to CDC and reconfirmed as positive for *Cyclospora* oocysts. Results of stool testing for other microbes were negative.

Table 1. Information on attendees of a wedding reception associated with an outbreak of cyclosporiasis, Pennsylvania, June 2000

| Variable | Case-patients (n=54)[a] | Non-case attendees (n=25)[b] |
|---|---|---|
| Age, y, median (range)[c] | 38.8 (18–87) | |
| Female sex, no. (%)[c] | 32 (59.3) | |
| Symptoms, no. (%) | | |
| Diarrhea[d] | 54 (100) | 1 (4.0) |
| Nausea | 43 (79.6) | 1 (4.0) |
| Stomach cramps | 41 (75.9) | 1 (4.0) |
| Chills/sweats | 41 (75.9) | 0 |
| Headache | 36 (66.7) | 0 |
| Muscle/body aches | 36 (66.7) | 1 (4.0) |
| Fever[e] | 32 (59.3) | 0 |
| Vomiting | 26 (48.1) | 0 |
| Dizziness | 25 (46.3) | 0 |
| Rash | 3 (5.6) | 0 |
| Hospitalization, no. (%) | 2 (3.7) | 0 |
| Length of stay, days, median (range) | 12 (10–14) | 0 |

[a] Five of the 54 case-patients had laboratory-confirmed cyclosporiasis.
[b] Two of the 25 non-case attendees reported having at least one symptom.
[c] Information about age and sex was obtained from only two non-case attendees and was not included in the table.
[d] Diarrhea was defined as having three or more loose stools in a 24-hour period.
[e] In case-patients, the median temperature was 37.8°C (range 37.2–38.5°C). No non-case attendees were febrile.

PCR analyses conducted by both FDA and CDC confirmed the presence of *Cyclospora* DNA in the raspberry filling of the cake. At FDA, an FTA-based PCR identified *Cyclospora* DNA in the raspberry filling but not in the icing from the top of the cake or in the cake itself. At CDC, PCR amplification was possible from DNA directly extracted from four of the nine portions of the raspberry filling that were tested. All positive samples generated sequences that were identical to *C. cayetanensis* in the 636-bp PCR product.

### Environmental and Source Investigations

None of the catering staff reported having been ill during the 3 weeks before or after the reception. However, on July 18, the local health department learned, through routine surveillance, about a caterer, who had worked during the reception, in whom gastrointestinal illness developed 8 days later; she had laboratory-confirmed cyclosporiasis. Although she could not recall her duties for the event, her usual tasks included cutting and serving wedding cake. She also identified two other staff members in whom gastrointestinal illness developed a median of 6 days after the reception; their symptoms resolved spontaneously, and no stool specimens were tested. They had not worked during the reception but had worked later that day and reported that they probably ate leftover cake.

Raspberries were the only produce used in the wedding cake. The catering company had received four shipments of fresh raspberries June 1–3. Some of the raspberries from these shipments were served fresh at events on June 3–4. The leftover raspberries were frozen for 1–4 days. Two to three pints of leftover, unwashed raspberries were thawed, crushed into pieces, and folded into the butter cream filling used in the cake. The cake was then frozen for another 4–5 days until it was thawed and eaten on June 10, the date of the wedding (Figure 2).

Local distributors A and B supplied the four shipments of raspberries received by the catering company during the period June 1–3 (Figure 3). Distributors A and B had received shipments from three other local distributors (C, D, and E) during May 30–June 3. During this period, one Guatemalan farm and one Mexican farm were the sources of the raspberries that were delivered to importers F and G that supplied distributor C, and an unknown number of U.S. farms supplied the raspberries delivered to distributors D and E (Figure 3). Which raspberries from which shipment were actually used in the cake is unknown.

The one Guatemalan farm that could have supplied the raspberries used in the cake was also one of the possible sources (and the only Guatemalan source) of raspberries served at a bridal brunch in Georgia in May 2000 that was associated with an outbreak of cyclosporiasis (14). The Guatemalan farm was the only known source in common to the events in Pennsylvania and Georgia. FDA inspected this farm and found no definitive source for contamination (J. Guzewich, pers. comm).

Table 2. Food items at a wedding reception that were significantly associated with cyclosporiasis in univariate analyses, Pennsylvania, June 2000

| Food items[a] | Attack rate in attendees, no. ill/no. exposed or unexposed (%) | | RR (95% CI) |
|---|---|---|---|
| | Exposed | Unexposed | |
| Wedding cake[b] | 50/53 (94.3) | 4/26 (15.4) | 6.1 (2.5 to 15.1) |
| Fresh fruit[c] | 36/43 (83.7) | 18/36 (50.0) | 1.7 (1.2 to 2.4) |
| Arugula salad | 34/41 (82.9) | 20/38 (52.6) | 1.6 (1.1 to 2.2) |
| Focaccia bread | 15/16 (93.8) | 39/63 (61.9) | 1.5 (1.2 to 1.9) |
| Hearthbaked bread | 23/27 (85.2) | 31/52 (59.6) | 1.4 (1.1 to 1.9) |

[a]Mesclun lettuce was served as a garnish on several hors d'oeuvres trays, and basil was served fresh in one food item. Neither was significantly associated with illness.
[b]Food item was statistically significant in multivariate analyses (relative risk, 5.9; 95% confidence interval [CI] 3.6 to 10.5).
[c]Fresh fruit included strawberries, raspberries, blueberries, and blackberries. Persons served themselves from a bowl of fresh strawberries and a bowl of fresh raspberries, blueberries, and blackberries next to the cake. The raspberries in the bowl came from a different source than the raspberries in the cake filling and were not statistically significant in multivariate analyses.

## Discussion

We investigated an outbreak of cyclosporiasis associated with a wedding reception in Philadelphia in June 2000. The epidemiologic evidence and the identification by PCR analyses of *Cyclospora* DNA in the food item most strongly associated with illness indicated that the outbreak was foodborne. This is the first outbreak associated with raspberries for which *Cyclospora* DNA has been identified in the epidemiologically implicated food item. Because of the outbreak, the Philadelphia health department subsequently made cases of *Cyclospora* infection reportable.

We do not know whether the outbreak involved more cases in Pennsylvania than the cluster of cases we investigated. The catering company for the wedding reception received four shipments of fresh raspberries the weekend before the cake was prepared; which raspberries from which shipment were used for the reception rather than the other six events during the period of interest at which raspberries were served is unknown. We did not identify any more cases of cyclosporiasis by contacting the other six groups. However, perhaps only one of the shipments of raspberries and only some of the raspberries in that shipment were contaminated. The same Guatemalan farm was one of the possible sources of the raspberries (and the only Guatemalan source) served at the reception in Pennsylvania in June 2000 and the bridal brunch in Georgia in May 2000. The identification of only one farm in common to the two events suggests that the events were related.

The year 2000 was the fifth year since 1995 (i.e., 1995, 1996, 1997, 1998, and 2000) that outbreaks of cyclosporiasis occurred in the spring in the United States or Canada that definitely or probably were associated with Guatemalan raspberries (7). However, the recent outbreaks have been much smaller than the multistate outbreaks in 1996 and 1997 (7). After the outbreaks in 1996 and 1997 (2,3), FDA began working with the Guatemalan government and berry industry to improve farming and exporting practices for raspberries. Only farms that meet certain standards—including water, sanitation, and worker hygiene issues—have been allowed to export fresh raspberries to the United States during the "spring season" (March through August). The standards are reviewed and updated yearly. During the spring of 2000, five Guatemalan farms were allowed to export to the United States. After the outbreaks in Pennsylvania and Georgia, FDA did not allow the farm that was in common to the events to export raspberries to the United States during the spring of 2001. No U.S. outbreaks of cyclosporiasis associated with Guatemalan raspberries were identified that spring. During the spring of 2002, only three

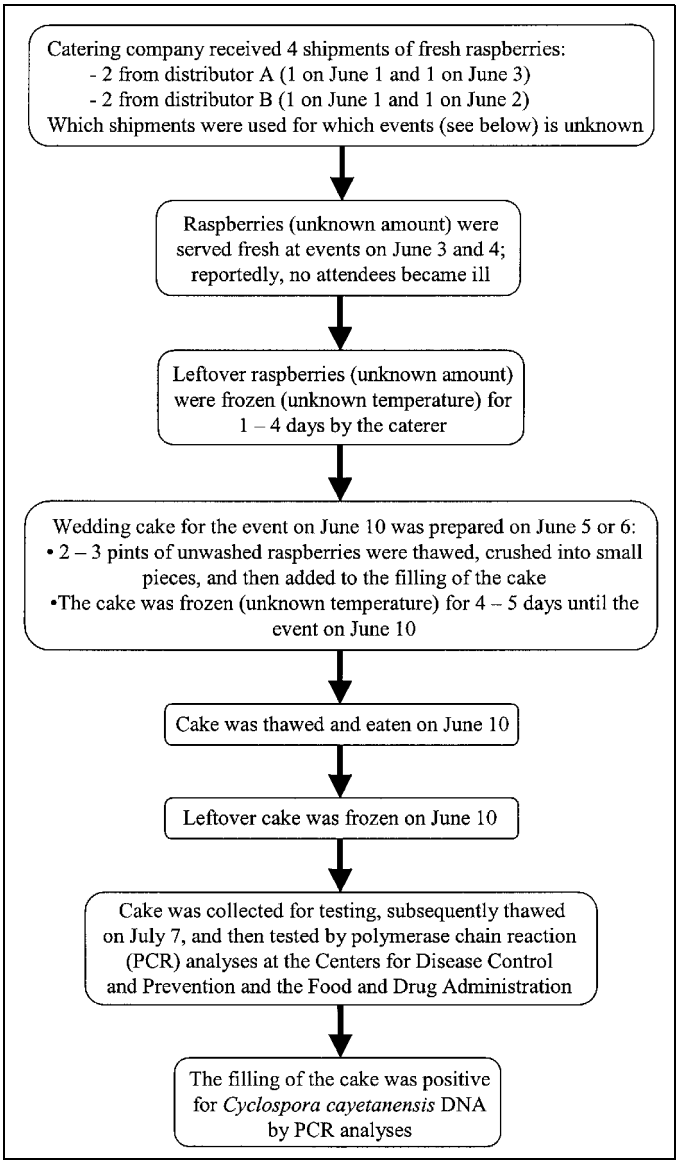


Figure 2. Flow chart with details about raspberries and wedding cake served at a wedding reception in June 2000 in Pennsylvania. The shipment of raspberries the catering company received on June 3 included raspberries from Guatemala. As noted, some details (e.g., the date the cake was prepared, the temperature of the freezer at the catering company) are unknown.

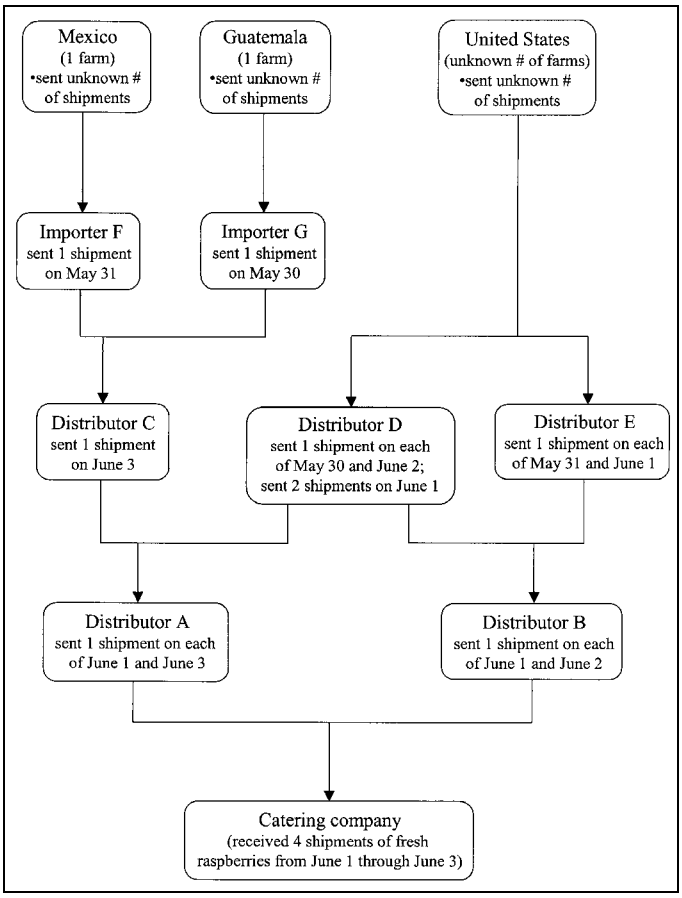


Figure 3. Flow chart with details about the possible sources of the raspberries used in the wedding cake served at a wedding reception in June 2000 in Pennsylvania. The Guatemalan farm was also a possible source of raspberries served at an event in Georgia in May 2000. This farm was the only known source in common to these two events.

farms, which have never been implicated in outbreaks of cyclosporiasis, were allowed to export raspberries to the United States.

The cyclosporiasis outbreak in Philadelphia is the first for which raspberries that reportedly had been frozen, rather than served fresh, were implicated. The raspberries used in the wedding cake had been bought fresh but then reportedly frozen and thawed twice before they were served (Figure 2). The temperature of the freezer where the raspberries were stored is unknown. When the freezer was inspected 2 weeks after the outbreak, the temperature of the freezer was -3.3°C, which might not have been cold enough to kill *Cyclospora* oocysts. Although limited information is available about the effects of freezing on their survival, for *Cryptosporidium parvum*, another coccidian parasite, oocysts remain infectious for mice after storage at –10°C for 1 week (15). Freezing at sufficiently cold temperatures for sufficiently long periods, as during commercial freezing, should kill *Cyclospora* oocysts. For *Cryptosporidium parvum,* oocysts were not infectious for mice after storage at –15°C for 1 week (15).

This Pennsylvania outbreak is the second documented U.S. outbreak, and the first associated with raspberries, for which *Cyclospora* has been detected in the epidemiologically implicated food item. The first U.S. outbreak for which *Cyclospora* was detected in a food item occurred in 1999 in Missouri and was associated with fresh basil in a chicken pasta salad (8). Neither the raspberries in 2000 nor the basil in 1999 had been washed. Although all produce should be thoroughly washed before being eaten, other outbreaks (2,3,7) and a laboratory study (16) have shown that washing does not eliminate the risk for *Cyclospora* infection.

Fortunately, leftovers from both the Pennsylvania and Missouri outbreaks had been frozen and were available for testing. Contamination of the implicated food items for the 1999 outbreak was demonstrated by PCR analyses and light microscopy and for the 2000 outbreak by PCR analyses. The modes of contamination of the produce associated with outbreaks of cyclosporiasis have not yet been definitively identified for any of the outbreaks. However, molecular methods are sensitive tools that can be used to identify the organism in the vehicle and in environmental samples, which then may provide information useful for determining the mode of contamination. Molecular methods for detecting *Cyclospora* continue to improve (9). Continued research about the biology and epidemiology of *Cyclospora* is important for identifying effective measures for preventing future outbreaks.

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# Human Exposure to Herpesvirus B–Seropositive Macaques, Bali, Indonesia

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Herpesvirus B *(Cercopithecine herpesvirus 1)* has been implicated as the cause of approximately 40 cases of meningoencephalitis affecting persons in direct or indirect contact with laboratory macaques. However, the threat of herpesvirus B in nonlaboratory settings worldwide remains to be addressed. We investigated the potential for exposure to herpesvirus B in workers at a "monkey forest" (a temple that has become a tourist attraction because of its monkeys) in Bali, Indonesia. In July 2000, 105 workers at the Sangeh Monkey Forest in Central Bali were surveyed about contact with macaques (*Macaca fascicularis*). Nearly half of those interviewed had either been bitten or scratched by a macaque. Prevalence of injury was higher in those who fed macaques. Serum from 31 of 38 Sangeh macaques contained antibodies to herpesvirus B. We conclude that workers coming into contact with macaques at the Sangeh Monkey Forest are at risk for exposure to herpesvirus B.

Herpesvirus B (*Cercopithecine herpesvirus 1* [CeHV-1]) is an alphaherpesvirus endemic to macaques of South and Southeast Asia (1). In macaques, the usual host, CeHV-1 causes mild symptoms, similar to the effects of herpes simplex virus 1 in humans (1). Clinical findings in macaques usually consist of oral or perioral vesicular lesions. After initial infection, CeHV-1 remains latent in the dorsal root or trigeminal ganglia of an infected macaque and can be shed periodically through herpetic lesions.

In contrast to its benign course in macaques, in humans CeHV-1 produces a fulminating meningoencephalitis with a mortality rate approaching 70% (2). Since first reported in the 1930s, a total of 43 cases of CeHV-1 have been diagnosed worldwide, all reported from the United States, Great Britain, or Canada, exclusively in people who had direct or indirect contact with laboratory macaques (2–6).

Several modes of primate-to-human transmission have been implicated, most involving direct exposure of tissue or fluid from an infected macaque. Weigler's 1992 review of human CeHV-1 cases (1) found that most were infected through direct bite and scratch wounds: one case resulted from direct contamination of a preexisting wound with monkey saliva, two cases occurred after lacerations from culture bottles containing macaque cells, and two occurred in persons punctured by needles previously used in macaques. One case of human-to-human transmission has been documented, when infection developed in the wife of a man who subsequently died of a CeHV-1 infection. She had a rash on her finger that came into contact with a vesicular lesion on her husband's arm, at the site of a monkey bite. The most recent documented case occurred in 1997 at the U.S. Yerkes Regional Primate Center, where a young worker who received an ocular exposure with contaminated body fluids from a CeHV-1–positive macaque became ill and subsequently died (6).

Two published case series have studied transmission of CeHV-1 from primates to humans. Friefeld et al. (7) examined prevalence of antibodies to CeHV-1 in primate handlers exposed to bites, scratches, needle-sticks, and mucosal splashes from laboratory macaques. None of the 166 exposed persons had antibodies to the virus. Similarly, in a small study of eight persons bitten by pet macaques, none seroconverted (8). Nevertheless, the threat of herpesvirus B has led the Centers for Disease Control and Prevention to recommend strict precautions for persons who come into contact with monkeys in occupational settings (2,6,9,10).

The threat of CeHV-1 to humans in nonlaboratory contexts worldwide has yet to be studied, despite the fact that the laboratory macaques that harbor the virus originated in Asia or are descendents of macaques originating there. Macaque species range throughout South and Southeast Asia and have adapted well to human-altered environments. In turn, macaques have become incorporated into religious mythology and local culture. Hindus in Indonesia, Nepal, and India, for example, regard macaques as sacred (11), and in many areas protected macaque populations have thrived alongside dense human settlements for centuries. On the Indonesian island of Bali, more than 44 Balinese Hindu temples have, over the centuries, become refuges for populations of free-ranging macaques (11). These monkeys subsist at least in part on the food and flower offerings left by Balinese Hindu worshipers. Over time, some of these temples have become tourist destinations known as "monkey forests," where macaques are the premier attraction. At the Sangeh Monkey Forest in Central Bali, dozens of local photographers make a living by enticing macaques to

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climb onto visitors and selling the photos of visitors posing with macaques. However, photographers and tourists are by no means the only humans who come into contact with these macaques. At Sangeh, three troops of macaques, a total of >200 monkeys, range throughout the monkey forest, along a road lined with merchants' shops, and into the adjacent town. Their daily travels afford ample opportunity for contact with shop owners and others who pass near the monkey forest.

This study investigated human-macaque contact in people who work in and around the Sangeh Monkey Forest. Our aim was to examine the risk of exposure to CeHV-1 from Sangeh's macaques. We addressed two principal questions: 1) does human-primate contact capable of transmitting CeHV-1 occur in this context? and 2) are the Sangeh macaques seropositive for antibodies to this virus?

## Methods

### Study Site

The Sangeh Monkey Forest is located in the Abiensemal District, central Bali, approximately 20 km north of Denpasar, Bali's most populous city. Built in the 17th century by the royal family of Mengwi, it is now maintained by the people of the village of Sangeh. The macaque (*Macaca fascicularis*) population at Sangeh totals approximately 200 animals. Their range extends through the monkey forest proper and across a main road that abuts the area. The monkey forest, a 6-hectare stand of *Dipterocarpus hasseltii* and other climax forest trees with heights up to 40 m, actually provides little food for the macaques. Most of their caloric intake is from daily provisions provided by temple workers and food given to them by visitors.

Visitors interact with macaques mainly in an open area adjacent to the principal temple structures. The main entrance to the monkey forest is reached by a promenade lined by shops offering clothing and souvenirs, in addition to peanuts and bananas for the macaques. Groups of macaques also make their way down this thoroughfare or along the shop roofs during their daily ranging.

### Ethnographic Surveys

The local temple committee provided researchers with a list of 250 persons whose work in and around the monkey forest brought them into regular proximity with the macaques. Most persons were either photographers or merchants whose shops lined the road leading to the monkey forest. Of this group, 105 persons (42%) volunteered to participate in the study. In July 2000, a questionnaire that focused on human-primate contact, written in Bahasa Indonesia, the national language of Indonesia, was administered by Balinese team members. Information requested included the type and number of contacts with Sangeh's macaques, injuries or sequelae resulting from macaque bites and scratches, and treatments of those injuries.

### Field Protocol

Universal precautions were observed during animal handling and specimen collection to minimize the risk of pathogen transmission between researchers and nonhuman primate subjects. All methods were reviewed and approved by the University of New Mexico's Institutional Animal Care and Use Committee.

Macaques were opportunistically darted within the monkey temple area and surrounding forest by using a Pneu-Dart air-powered pistol (Pneu-Dart Inc., Williamsport, PA). Darts were loaded with 15 mg of Telazol (Fort Dodge Laboratories, Fort Dodge, IA; tiletamine HCl/zolazepam HCl) to ensure initial sedation. Immediately after darting, the macaque was moved to a secluded area and <5 mg/kg of supplemental Telazol was administered for sedation. Six milliliters of blood was withdrawn from the femoral vein, placed in a serum separator tube, and centrifuged in the field to extract the serum. Sera were frozen and stored at –20°C. Dental eruption sequence was recorded and used as a proxy measure of chronologic age. Macaques were observed and allowed to recover from anesthesia in a quiet area before being released. No macaques were injured as a result of this protocol.

### Laboratory Techniques and Data Analysis

Enzyme-linked immunosorbent assays (ELISA) to detect antibodies to CeHV-1 in macaque sera were performed at the B-Virus Reference Laboratory at Georgia State University (12). Questionnaire and serologic data were entered into a spreadsheet, and univariate analysis was performed with the JUMP-IN 4 statistical software package (SAS Institute, Inc., Cary, NC, version 4). The association between macaque CeHV-1 seropositivity and age was determined by chi-square test. Prevalence ratios, regarded by some as the most appropriate tool for analyzing cross-sectional studies, were calculated to describe associations between demographic variable and feeding behavior and prevalence of bite and scratch exposure (13,14). For all variables the category with the lowest prevalence was used as the referent. Calculation of prevalence ratios and 95% confidence intervals [CI] was performed with the NCSS Statistical Software package (Kaysville, UT).

## Results

### Seroprevalence of Antibodies to CeHV-1 in Macaques

Demographic and serologic data on the macaques sampled are shown (Table 1).

Thirty-one (81.6%) of the 38 sampled macaques tested positive for antibodies to CeHV-1. One (25%) of the four juveniles, two (33.3%) of the six subadults, and all 28 adults (100%) were seropositive. The association of increased seroprevalence with increasing age was statistically significant ($p<0.0001$, chi square). These figures are consistent with those of other seroprevalence studies performed on captive and noncaptive macaques (15–18).

Table 1. Seroprevalence of antibodies to *Cercopithecine herpesvirus 1* (herpesvirus B) in Sangeh macaques (*Macaca fascicularis*), Bali

| Age group[a]/sex | No. (%) ELISA positive/total |
|---|---|
| **Juvenile** | |
| Male | 1/4 (25) |
| Female | — |
| Total | 1/4 (25) |
| **Subadult** | |
| Male | 1/4 (25) |
| Female | 1/2 (50) |
| Total | 2/6 (33.3) |
| **Adult** | |
| Male | 21/21 (100) |
| Female | 7/7 (100) |
| Total | 28/28 (100) |
| **All ages** | |
| Male | 23/29 (79.3) |
| Female | 8/9 (88.8) |
| Total | 31/38 (81.6) |

[a]Juveniles are defined as 1–3 years of age; subadults as 3–5 years of age; adults as >5 years of age.
—, no data; ELISA, enzyme-linked immunosorbent assay.

### Demographics of the Human Study Population

Demographic data for the human study participants are summarized in Table 2. The mean age of the study population was 35.6 years (standard deviation 12.0). The median age was 35 years (range 18–75); 63.8% of respondents were male. The three most common occupations were merchants (34.3%), photographers (24.8%), and farmers (18.1%). Consistent with cultural norms, 35 of the 36 merchants were women who owned shops along the main promenade leading to the temple area. Of the 38 female respondents, all but 3 were merchants. All respondents were Balinese Hindus residing in the village of Sangeh.

### Bite and Scratch Results

Prevalences of bite and scratch injuries are summarized in Table 3. Results from the survey showed that 76.9% of persons had touched or been touched by a macaque. Almost a third (29.5%) reported that they had been bitten on at least one occasion by a macaque; 39% had been scratched at least once; some persons reported being both bitten and scratched. Of the 51 injured, 94.1% were holding food at the time of the injury.

### Anatomic Distribution of Injuries, Sequelae, and Wound Treatment

By anatomic distribution, 64.7% of the 51 injuries were to the hands, 7.8% to the arms, 7.8% to the legs, 11.8% to the head, and 7.8% to the back or buttocks. Of the 51 persons bitten or scratched, 51% reported that the wound had bled, 9.8% reported a rash at the bite site, 11.8% noted fever after their injury, 3.9% had headache, and 5.9% had generalized weakness. None of those interviewed reported symptoms consistent with previously described cases of CeHV-1 infection.

In terms of wound treatment, 54.9% of those injured did not use any kind of treatment for their scratch or bite injuries, 21.6% washed the wound with an antiseptic solution, and 19.6% washed with soap. Of the injured, 11.7% were treated at a medical clinic, and 2% were treated by an herbalist. Five of the persons treated at the medical clinic received antibiotics; none received antiviral prophylaxis.

### Prevalence Ratios for Exposures to Monkey Bites and Scratches

Table 4 presents prevalence and prevalence ratios for exposure to monkey bites or scratches, by respondent's age

Table 2. Demographic characteristics of human study participants, Sangeh, Bali

| Demographic characteristic | No. (% of total) |
|---|---|
| All persons | 105 (100) |
| **Age group** | |
| <20 | 11(10.5) |
| 20–29 | 27 (25.7) |
| 30–39 | 26 (24.8) |
| 40–49 | 31 (29.5) |
| >49 | 10 (9.5) |
| **Sex** | |
| Male | 67 (63.8) |
| Female | 38 (36.2) |
| **Marital status** | |
| Single | 29 (27.6) |
| Married | 75 (71.4) |
| Widowed | 1 (1) |
| **Education** | |
| ≤6th grade | 38 (36.2) |
| 7th–9th grade | 23 (21.9) |
| 10th–12th grade | 38 (36.2) |
| University | 6 (3.8) |
| **Occupation** | |
| Merchant | 36 (34.3) |
| Photographer | 26 (24.8) |
| Farmer | 19 (18.1) |
| Security | 7 (6.7) |
| Government employee | 5 (4.8) |
| Traditional guard | 5 (4.8) |
| Laborer | 4 (3.8) |
| Other | 3 (2.9) |

Table 3. Prevalence of bite and scratch injuries from monkeys to humans, Sangeh, Bali

| Descriptor | No. (%) of persons (n = 105) |
|---|---|
| Bitten | 31 (29.5) |
| Bitten more than once | 7 (6.7) |
| Scratched | 41 (39.0) |
| Scratched more than once | 15 (14.3) |
| Bitten or scratched | 51 (48.6) |
| Possessed food at time of injury | 48 (94.1) |
| All persons | 105 (100) |

group, sex, occupation, level of education, and whether the respondent fed macaques. Persons in their twenties had the highest prevalence of exposure (63%) while those <20 years of age had the lowest (9%). The exposure rate for male participants (62.7%) was higher than that for females. The prevalence ratio for males was 2.6, indicating that their exposure was more than two and a half times as common as that of female study participants. Of occupations represented by more than five persons, farmers (94.7%) had the highest exposure prevalence, followed by photographers (57.7%), merchants (25%), and security guards (14.3%). For farmers, the prevalence ratio was 6.6, with a 95% CI (1.61, 78.46). Exposure rates were higher in persons with grade school or less education (57.9%) and middle school education (56.5%) than those who had reached high school (39.5%) and university (16.7%).

Most men (89.4%) and women (79%) reported that they had offered food to macaques. Injury was more prevalent in persons who reported feeding macaques (55.6%) than in those who denied feeding them (7.1%). The prevalence ratio in those who fed monkeys was 8.3, indicating that exposure in those who fed monkeys was nearly eight times more common than in those who did not feed monkeys.

## Discussion

### Exposure to Macaque Bites and Scratches at the Sangeh Monkey Forest

The survey data presented in this study suggest that many workers in and around the Sangeh Monkey Forest have been bitten or scratched by a macaque. Serologic data show that >80% of these macaques have been exposed to CeHV-1. Current understanding of the pathophysiology of this virus predicts that seropositive animals periodically shed it through mucosal lesions (1,17–19). Therefore, these workers report injuries that put them at risk for exposure to the virus.

### Wound Care and the Risk for Pathogen Transmission

In contrast with laboratory settings, where protocols regarding care of nonhuman primate-inflicted wounds specify immediate and thorough decontamination, awareness of the risk of zoonotic disease in workers at Sangeh is low. Data on care of macaque bite and scratch wounds reflected this. Lack of prompt and rigorous wound care may thus pose added risk for transmission of CeHV-1 and other nonhuman primate-borne pathogens for workers and visitors at Sangeh.

### CeHV-1 a Cause of Human Disease on Bali?

The above data suggest that human-macaque contact capable of transmitting CeHV-1 is relatively common at Sangeh. Indeed, these data may represent only a small fraction of the human-macaque contact occurring there. Wheatley (11) reported that up to 40% of visitors to Sangeh are bitten by a macaque. Given that thousands of tourists visit Sangeh during a typical month, a reasonable estimate of the annual number of injuries inflicted by macaques is in the thousands, and Sangeh is but one of a handful of monkey forests on Bali that draw large numbers of visitors. Yet no case of human CeHV-1 infection has been reported in Bali, either in association with monkey forests or in any other nonlaboratory context (K. Suaryana, pers. comm.). Several explanations for this observation can be offered.

Recent work supports the existence of three genotypes of CeHV-1, each associated with a distinct species of macaque (20). It has been suggested that only the strain associated with rhesus macaques (*M. mulatta*), the most commonly used laboratory macaque, causes virulent disease in humans (20). This hypothesis is based on the observation that, when the identity of the source animal was known, human CeHV-1 infection was associated with exposure to rhesus macaques but never solely with exposure to other macaque species, including *M. fascicularis*, the species found in Bali, and the second-most commonly used macaque in laboratory research.

The hypothesis that only certain species of macaques may carry a pathogenic strain of CeHV-1 has not been tested. Almost all such infections have occurred in laboratory settings where rhesus macaques constitute most of laboratory nonhuman primates. In addition, rhesus macaques tend to be the more aggressive species. Thus, rhesus macaques may simply cause more injuries and hence be associated with more viral exposures than other macaque species. Furthermore, no case of CeHV-1 infection in humans has been reported in India or Nepal, countries where human-macaque contact is known to occur outside the laboratory and where the predominant macaque species is the rhesus monkey. However, no active surveillance for CeHV-1 is carried out in Nepal and India, and persons diagnosed with encephalitis in these countries are highly unlikely to be tested for this virus.

Another possible explanation for the lack of reported human CeHV-1 cases is that opportunities for exposure to actual virus may be rare. Previous research suggests that, in the laboratory setting, macaques seropositive for CeHV-1 antibodies, even under certain kinds of stress, infrequently shed the virus (17,19). If the macaques at Sangeh behave similarly and shed the virus infrequently, the opportunity for exposure may be rare. One must also take into account the probability that the macaques that bite or scratch carry CeHV-1. Specifically, infant and juvenile macaques are less likely to test posi-

Table 4. Prevalence and prevalence ratios for exposure to monkey bites or scratches by different variables, Sangeh, Bali

| Variable | Total no. (%) persons exposed | Prevalence ratio | 95% CI | p value |
|---|---|---|---|---|
| **Age group (yrs)** | | | | |
| <20 | 11 (9.0) | 1.0 | — | — |
| 20–29 | 27 (63.0) | 6.9 | 1.75, 117.30 | 0.045 |
| 30–39 | 26 (50.0) | 5.5 | 1.34, 93.86 | 0.080 |
| 40–49 | 31 (45.2) | 5.0 | 1.21, 84.81 | 0.100 |
| >49 | 10 (60.0) | 6.0 | 1.47, 113.93 | 0.056 |
| **Sex** | | | | |
| Female | 38 (23.7) | 1.0 | — | — |
| Male | 67 (62.7) | 2.6 | 1.55, 5.26 | 0.002 |
| **Occupation** | | | | |
| Security | 7 (14.3) | 1.0 | — | — |
| Merchant | 36 (25.0) | 1.7 | 0.41, 29.67 | 0.564 |
| Photographer | 26 (57.7) | 4.0 | 1.08, 66.80 | 0.138 |
| Farmer | 19 (94.7) | 6.6 | 1.90, 108.43 | 0.041 |
| Other | 17 (47.1) | 2.2 | 0.80, 55.40 | 0.215 |
| **Education** | | | | |
| University | 6 (16.7) | 1.0 | — | — |
| High school | 38 (39.5) | 2.4 | 0.64, 38.91 | 0.356 |
| Middle school | 23 (56.5) | 3.4 | 0.93, 55.53 | 0.190 |
| Grade school or none | 38 (57.9) | 3.5 | 0.99, 56.51 | 0.177 |
| **Fed monkeys** | | | | |
| No | 15 (6.7) | 1.0 | — | — |
| Yes | 90 (55.6) | 8.3 | 2.08, 142.05 | 0.029 |

CI, confidence intervals.

tive for anti-CeHV-1 antibodies than older macaques and are thus, as a group, probably less likely to shed virus (1,15). Data from the Ubud Monkey Forest (A. Fuentes, pers. comm.) suggest that adult male and female macaques accounted for approximately half of all bites, with the remainder attributed to juveniles. At least half the bites, therefore, are caused by macaques that are less likely to harbor the virus. Unfortunately, no large-scale studies of CeHV-1 shedding in wild macaques have been performed to date, so the rate at which these animals shed the virus is unknown.

CeHV-1 infection in humans might also be underreported if the disease is rare, especially since awareness of this virus among health-care providers in Bali is low. The symptoms of infection might be mistaken for those of other neurologic diseases, such as polio or Guillain-Barré syndrome. Thus it is theoretically possible that humans reporting mild sequelae following monkey bites and scratches might be describing a mild variant of B virus syndrome. We are aware of no research examining the prevalence of antibodies to CeHV-1 in persons with neurologic syndromes in Asia.

Finally, resistance to CeHV-1 in the exposed human population may explain the lack of reported cases. Human populations living in proximity to the monkey forests in Bali have been living commensally with macaques for centuries. These populations could conceivably acquire immunologic resistance to the virus as a result of frequent exposure over time.

More data are needed to assess whether CeHV-1 poses a substantial public health threat to workers at Sangeh. Serologic data from humans who have been scratched or bitten by macaques could help to determine whether these persons have been exposed to the virus. In addition, a thorough search for cases of human CeHV-1 infection, in Southeast Asia as well as in South Asia, could yield further insight into the epidemiology of this virus in the human population. However, the virus has not surfaced as a recognized infectious threat for humans in areas where the two species have lived commensally for centuries.

### Public Health Significance of Nonhuman Primate Zoonoses

Data such as those presented in this study can play an important role in preventing the emergence of primate zoonoses. By understanding where and how interspecies contact occurs we may take informed steps toward reducing the likelihood of interspecies pathogen transmission. Specifically, information about interspecies pathogen transmission may help to identify priority areas for intervention to reduce the emergence of nonhuman primate-borne zoonoses.

### Implications and Recommendations for Public Health Practice

Over the past decades, the widespread use of laboratory nonhuman primates as models for the study of human diseases has led to the exposure of laboratory workers to infectious agents endemic in nonhuman primates. Relatively little is known about the epizootology of infectious agents harbored by nonlaboratory macaques and even less is known about the transmission of these agents from macaques to humans with whom they come into contact. CeHV-1 is one of a few infectious agents known to be transmitted from macaques to humans. Serologic, virologic, and molecular studies have demonstrated that a handful of laboratory workers have acquired infection with *Simian foamy virus* as well as *Simian immunodeficiency virus* and simian retrovirus, though no known adverse health effects were associated with these infections (21–29). Very little is known about the effects of these viruses on humans, since the number of seroconverters is low, <10 cases for each virus. No serologic studies outside laboratory settings have been conducted. Given the paucity of data on the effects of CeHV-1 and other endemic macaque pathogens on humans, especially in nonlaboratory settings, reducing the kinds of interspecies contact most likely to lead to pathogen transmission would be prudent. Our data suggest some possible interventions. Because workers who fed macaques were far more likely to be bitten or scratched than their other col-

leagues, an intervention aimed at reducing injuries in workers might logically focus on feeding practices. Restricting feeding to specially trained personnel who distribute food to macaques in such a manner as to avoid physical contact with them is one strategy that has worked effectively at other monkey forests (A. Fuentes, pers. comm.). Also, since approximately two thirds of those who are injured report injuries to the hands, the use of protective gloves should be advocated for personnel coming into frequent contact with monkeys. Of course, implementing these kinds of changes would require a commitment from the community to change the way the monkey forests operate.

Another incentive for enacting such changes is that monkey forests and the macaques that live in them are valuable cultural and economic resources to the communities in which they are located. Disease transmission in the opposite direction, namely human to nonhuman primate, may threaten these macaques. If so, regulating interspecies contact could help to preserve the monkey forests as an economic resource for the community. Perhaps a long-term strategy to preserve monkey forests will recognize the importance of minimizing infectious risks to both humans and macaques.

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# DNA Vaccine Expressing Conserved Influenza Virus Proteins Protective against H5N1 Challenge Infection in Mice

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Influenza vaccination practice, which is based on neutralizing antibodies, requires being able to predict which viral strains will be circulating. If an unexpected strain, as in the 1997 H5N1 Hong Kong outbreak, or even a pandemic emerges, appropriate vaccines may take too long to prepare. Therefore, strategies based on conserved influenza antigens should be explored. We studied DNA vaccination in mice with plasmids expressing conserved nucleoprotein (NP) and matrix (M) from an H1N1 virus. After vaccination, mice were challenged with A/H5N1 viruses of low, intermediate, and high lethality. A/NP+A/M DNA vaccination reduced replication of A/Hong Kong/486/97 (HK/486), a nonlethal H5N1 strain, and protected against lethal challenge with more virulent A/Hong Kong/156/97 (HK/156). After HK/156 exposure, mice survived rechallenge with A/Hong Kong/483/97 (HK/483), although the DNA vaccination alone protected poorly against this highly virulent strain. In the absence of antigenically matched hemagglutinin-based vaccines, DNA vaccination with conserved influenza genes may provide a useful first line of defense against a rapidly spreading pandemic virus.

The 1997 outbreak of H5N1 avian influenza in humans in Hong Kong (1,2) caused alarm because it involved highly pathogenic strains of an influenza subtype to which humans lack immunity. This outbreak led to fears about inability to control a pandemic if a new strain should spread efficiently from human to human. Although prevention by vaccination is more desirable than treatment after infection, conventional immunization strategies have major limitations.

Neutralizing antibodies are specific to subtype and often strain, so vaccination based on eliciting such antibodies requires accurate prediction of the viral strains that will circulate during the influenza season and leaves little time for vaccine preparation. Even with usual epidemic strains, difficulties and delays in the production of an adequate vaccine supply have occurred in some years (3). A rapidly developing pandemic would shorten the timeframe to identify the viral strain and prepare an antigenically matched vaccine, while the need to vaccinate an entirely naïve population would exacerbate vaccine production and supply issues. In addition, H5 vaccine candidates, either H5 recombinant protein or a conventional surface antigen vaccine prepared from apathogenic H5N3 virus, have shown suboptimal immunogenicity in human trials (4,5).

A recent report on the molecular basis for virulence of H5N1 viruses (6) was accompanied by an article that discussed related public health issues, in which Laver and Garman (7) addressed the problem of how to control pandemics and concluded that currently "the most promising first line of defense" is use of antiviral drugs. These drugs, however, reduce symptoms and duration of disease only partially (8), and their effectiveness during H5N1 infection is unknown. Laver and Garman further commented that various experimental vaccines, including DNA vaccines, may be more promising for pandemic control. These statements highlight the fact that additional approaches are needed to produce effective vaccines for H5N1 or other new subtypes (9).

Vaccines using conserved components of influenza A virus can induce protection against many influenza A strains, including those of divergent subtypes. Animal studies have demonstrated potent and long-lasting heterosubtypic immunity, that is, exposure to a virus of one subtype protects against challenge infection with another subtype (10–15). The mechanisms of heterosubtypic immunity are not completely understood but likely include both T-cell immunity, in particular CD8+ cytotoxic T-lymphocytes (CTL) (16,17) and CD4+ T cells (13), as well as antibodies to conserved epitopes (18). Heterosubtypic immunity has been reported in humans (19, 20), but its effectiveness and duration are unknown. Animal studies may show ways to optimize induction of heterosubtypic immunity, which could then be tested in humans. Heterosubtypic immunity induced by virus can protect against H5N1 infection in animals (21), and human T cells specific for antigens of an H1N1 virus, including nucleoprotein (NP) and matrix (M), can lyse target cells infected with H5N1 virus (22). In addition, exposure to H9N2 virus can induce heterosubtypic protection against H5N1 challenge in chickens (23) and mice (24).

DNA vaccination can target immune responses to epitopes that are highly conserved in influenza A viruses, while avoid-

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ing the risks of live-virus vaccines. We and others have previously shown that DNA constructs expressing conserved influenza proteins induce antibody and T-cell responses and protect against H3N2 heterosubtypic challenge (25–27). Both CD4+ and CD8+ T cells play roles in this protective immunity. DNA vaccination has also been studied in the H5N1 system, although largely with constructs expressing HA. DNA constructs expressing H5 HA can protect against lethal H5N1 challenge in mice (28). In lethal challenge experiments with chickens, an H5 HA construct protected fully and a construct expressing NP of an H5N8 virus protected partially (29,30). However, DNA vaccines expressing heterosubtypic antigens have not been studied in the H5N1 system.

Studies of challenge with H5N1 viruses from the 1997 Hong Kong outbreak must take into account their phenotypic diversity. While all these viruses were highly pathogenic in chickens, two main pathogenicity phenotypes were observed in mice (31,32). Viruses of the two types were studied for histopathology, viral titers in various tissues, and lethality in mice. The H3N2 viruses A/Udorn or X31 were used for comparison in some cases. Some isolates, represented by A/Hong Kong/483/97 (HK/483), were lethal even at modest doses, replicating in multiple organs, including the brain, liver, spleen, and kidney after intranasal administration (31), resulting in pathology of respiratory tissue and the heart, and producing immune effects (33). Other isolates, represented by A/Hong Kong/486/97 (HK/486), replicated only in the respiratory tract and were not lethal. One virus, A/Hong Kong/156/97 (HK/156), did not fit readily into either group, requiring higher doses to infect or kill mice in one of the studies (31) and showing some spread to nonrespiratory sites but more limited spread than was seen with HK/483 (31,32). HK/483 and HK/156, but not HK/486, were isolated from lethal infections in the original human cases.

In this study, we extended DNA vaccination based on conserved influenza components to heterosubtypic challenge with H5N1 virus. We investigated whether the broadly cross-reactive immunity induced by immunization of mice with DNA expressing NP and M from a mouse-adapted human H1N1 virus, A/Puerto Rico/8/34 (A/PR/8), could control infection with a range of H5N1 viruses.

## Materials and Methods

Plasmid VR1012 was obtained from Vical Inc. (San Diego, CA) under a Materials Transfer Agreement. Full-length influenza genes for NP and M of A/PR/8 were prepared and inserted into VR1012 as described previously (27). The plasmid B/NP expresses the full-length NP gene from B/Ann Arbor/1/86 (B/AA), derived from a baculovirus vector generated by Rota et al. (34) and subcloned into VR1012. Plasmid DNA was prepared and tested as described (27). Endotoxin levels were <1 EU/100 μg dose.

H5N1 viruses used in this study were HK/156, HK/483, HK/485, and HK/486 (31). Other viruses used were H1N1 virus A/PR/8; reassortant virus X-31 with surface glycoproteins of A/Aichi/2/68 (H3N2) and internal proteins of A/PR/8 virus; and B/AA. The A/PR/8 and X-31 stocks were mouse adapted by passage through mouse lungs. Virus stocks were propagated in the allantoic cavity of embryonated hen eggs at 37°C for 24 hr (H5N1 viruses) or 34°C for 48–72 hr (other viruses). Fifty-percent egg infectious dose ($EID_{50}$) titers and mouse infectious dose ($MID_{50}$) titers were determined by serial titration in eggs or mouse lungs, respectively, and calculated by the method of Reed and Muench (35). All experiments with infectious H5N1 viruses were conducted under BSL-3+ containment, including work in animals.

BALB/c female mice were purchased from the Division of Cancer Treatment, National Cancer Institute, Frederick, Maryland, or from Jackson Laboratories, Bar Harbor, Maine. DNA was injected intramuscularly, 100 μg/mouse of each plasmid, three times at 2-week intervals, starting at 6–7 weeks of age. Approximately 1 week after the last immunization, mice were shipped from the Food and Drug Administration to Centers for Disease Control and Prevention or U.S. Department of Agriculture, allowed to rest for approximately a week, challenged under containment conditions with $CO_2$ anesthesia, and monitored for weight loss and death. For viral titers, lung and brain tissues were collected 6 days postchallenge and frozen.

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (15) on plates coated with lysates of influenza virus–infected cells. Hemagglutination inhibition (HI) was performed by standard methods with sera pretreated with receptor-destroying enzyme (36).

Thawed tissues were homogenized in 1 mL of sterile phosphate-buffered saline. Clarified lung, brain, kidney, and nose homogenates were titrated for virus infectivity in 10-day-old embryonated eggs ($EID_{50}$) from initial dilutions of 1:10 (lungs and nose) or 1:2 (brain and kidney), with positive eggs identified by hemagglutination. Detection limits were $10^{1.2}$ $EID_{50}$/mL for lung and nose, and $10^{0.8}$ $EID_{50}$/mL for brain and kidney. Enzyme-linked immunospot assays (ELISPOT) for interferon-γ (IFN-γ) secreting cells were performed as described previously (37).

For CTL assays, splenocytes were restimulated in vitro and target cells prepared as described (38). CTL activity was measured by lactate dehydrogenase (LDH) release (CytoTox 96 Non-Radioactive Cytotoxicity Assay kit G170, Promega Corp., Madison, WI). Results were calculated as:

$$\% \text{ Lysis} = \frac{\text{Experimental-Effector Spontaneous-Target Spontaneous}}{\text{Target Maximum-Target Spontaneous}} \times 100$$

where target maximum represents target cells plus Promega lysis solution containing detergent. Maximum cytotoxicity occasionally exceeds 100% (Figure 1). The addition of targets may alter spontaneous release from effectors, or detergent lysis may differ from CTL-mediated lysis, but relative CTL activity was consistent.

## Results

Mice were immunized with a mixture of plasmids encoding A/NP+A/M, intended to provide greater protection than a

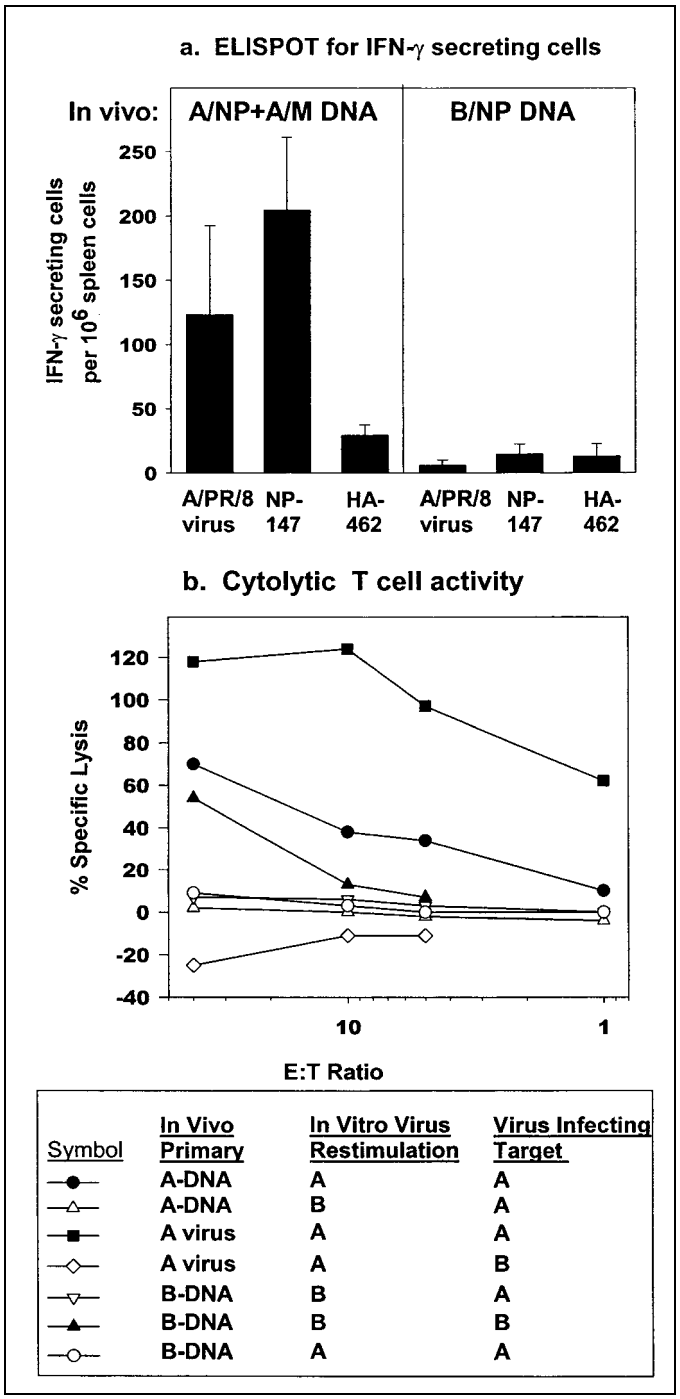


Figure 1. DNA vaccination induces T-cell responses. a) Enzyme-linked immuno spot (ELISPOT) assay for interferon-γ (IFN-γ) secreting cells. Mice were immunized three times with A/NP+A/M or influenza B nucleoprotein DNA (B/NP DNA) intramuscularly. Spleen cells were analyzed by ELISPOT, using peptides at 1 μg/mL or A/PR/8 live virus. Results are the mean of three experiments. No response to A/PR/8 virus occurred in one experiment. Concanavalin A (Con A) responses: A/NP+A/M groups, >274 for all experiments; B/NP groups, >329 for all experiments. b) Cytotoxic T-cell assay. Mice were vaccinated as above or with live A/PR/8 virus given on the day of the second DNA injection. Spleens were harvested 2½ weeks after the third DNA injection. Spleen cells were restimulated in vitro with live A/PR/8 or B/AA. After 7 days of culture, restimulated effector cells at various ratios were mixed with P815 target cells infected with A/PR/8 or B/AA, and lactate dehydrogenase (LDH) release measured.

single antigen (27,39). Plasmid DNA without an insert is often used as a control; although we used it initially, we later prepared a construct expressing the NP gene of influenza B/AA as a specificity control. The B/AA virus is only distantly related antigenically to influenza A, and no cross-protection is seen between influenza A and B viruses. The control plasmid expressing B/NP protects against challenge with influenza B, as shown by reduction in lung viral titers (40).

A/NP+A/M DNA induced antibodies against homologous A/PR/8 proteins (geometric mean ELISA titer 761), with no cross-reactivity to influenza B proteins (all titers <20). Mice immunized with B/NP DNA had comparable titers of antibody to influenza B proteins, with no cross-reactivity to A/PR/8 proteins.

DNA immunization activated T cells in an antigen-specific manner by two measures, ELISPOT of IFN-γ secreting cells and CTL activity. Splenocytes from mice immunized with A/NP+A/M DNA generated an IFN-γ ELISPOT response when restimulated with $NP_{147}$ peptide (the dominant CTL epitope in BALB/c mice), A/PR/8 virus, or concanavalin A (Con A), but not with control $HA_{462}$ peptide, demonstrating antigen specificity (Figure 1a). Mice immunized with B/NP DNA did not respond to restimulation with either peptide or with A/PR/8 but did respond to Con A, indicating the cells were functional.

Antigen-specific CTL responses to DNA immunization were seen after in vitro restimulation (Figure 1b). Cells from mice immunized with A/NP+A/M DNA lysed A/PR/8-infected targets if they had been restimulated with A/PR/8 but not with B/AA. Controls immunized with B/NP DNA and restimulated with B/AA generated cytolytic activity detectable on influenza B-infected targets but not A/PR/8-infected targets.

A/NP+A/M DNA immunization was tested for protection against an H5N1 challenge virus of low virulence, HK/486. HK/486 is not lethal for mice, so control of virus replication was measured. A/NP+A/M vaccination reduced replication of HK/486 virus in the lungs approximately 17-fold, compared with viral titers in mice vaccinated with control DNA or unimmunized mice (Table, highly significant by Analysis of variance (ANOVA); see legend). As expected, infection of mice with X-31 virus induced substantial heterosubtypic immunity, reducing lung virus titers by approximately 3,000-fold compared with unvaccinated controls.

Next, we tested the ability of A/NP+A/M DNA vaccination to protect against HK/156, an H5N1 challenge virus of intermediate virulence. Mice vaccinated with A/NP+A/M DNA had only minor weight loss after challenge, while mice vaccinated with control DNA lost weight dramatically (Figure 2a). Four of mice per group were euthanized at day 6 after challenge to measure virus replication in lungs and brains. A/NP+A/M DNA immunization reduced lung titers by over two logs (approximately 500-fold, highly significant by ANOVA, Figure 2b). As expected (21), immunization with A/PR/8 virus also reduced lung titers substantially. Reductions in brain titers

Table. Effect of DNA vaccination on replication of HK/486 challenge virus in mouse lungs[a]

| Immunization | No. mice | Lung titer +/- SE |
|---|---|---|
| Expt 1A | | |
| A/NP+A/M DNA | 6 | 5.7 ± 0.33 |
| B/NP + blank DNA | 6 | 6.9 ± 0.18[b] |
| None | 6 | 6.9 ± 0.22[c] |
| Expt 1B | | |
| Live X-31 virus | 4 | 3.6 ± 0.36[d] |
| None | 4 | 7.1 ± 0.1 |

[a]Mice were immunized intramuscularly with 100 µg each of influenza A nucleoprotein and matrix DNA (A/NP+A/M DNA) or controls with 100 µg each of influenza B nucleoprotein DNA (B/NP)+blank DNA (total dose 200 µg/mouse on each occasion), three times at 2-week intervals. Two weeks after the last dose of DNA, mice were challenged with 100 mouse infectious dose $(MID)_{50}$ of HK/486 intranasally. X31 virus-primed mice and their controls were challenged along with DNA-vaccinated mice. On day 6 after challenge, mice were sacrificed and lungs collected for titration of virus infectivity.
[b]Differs significantly from A/NP+A/M group by analysis of variation (ANOVA), p=0.0082.
[c]Differs significantly from A/NP+A/M group by ANOVA, p=0.011.
[d]Differs significantly from unimmunized group by ANOVA, p<0.001.

were not statistically significant because virus titers in the brain were low even in B/NP DNA-immunized controls.

Mice vaccinated (six per group) with A/NP+A/M DNA all survived a HK/156 challenge dose lethal to controls, as did A/PR/8-primed mice (Figure 2c). Thus, DNA vaccination with conserved components is effective not only against strains of low virulence like HK/486 but also against a lethal strain. However, A/NP+A/M DNA vaccination was not protective against challenge with 100 $MID_{50}$ of highly virulent A/HK/483 (none of six mice survived). An additional experiment used 100 $MID_{50}$ and a lower challenge dose of HK/483 to determine whether A/NP+A/M DNA vaccination could protect against a less extreme challenge. Challenge with 100 $MID_{50}$ of HK/483 again killed all the mice vaccinated with A/NP+A/M DNA (0/8 survived). With a challenge dose of 10 $MID_{50}$, four of eight mice vaccinated with A/NP+A/M DNA survived, but zero of eight given B/NP DNA and zero of eight naïve controls survived. These results suggest some protective effect, though the numbers are not statistically significant. Preliminary testing of viral titers in lung, nose, kidney, and brain at day 6 showed significant differences in the lungs and noses between A/NP+A/M immunized mice and controls, suggesting some impact of the immunization (data not shown).

A/NP+A/M DNA-vaccinated mice that survived HK/156 infection (above) were rechallenged 14 weeks later with 100 $MID_{50}$ of the virulent HK/483 strain. Since mice vaccinated with control DNA had all died after HK/156 challenge, a group of naïve animals was added to the HK/483 challenge to confirm lethality of the challenge dose. All mice primed with A/NP+A/M DNA and subsequently exposed to HK/156 survived this HK/483 challenge, whereas all naïve mice died by day 8 (Figure 2d).

Anti-HA (H5) antibodies induced by HK/156 exposure might account for the protection against HK/483 infection. To assess this possibility, we tested for HI reactivity in sera from the mice after HK/156 exposure but before HK/483 challenge. All mice immunized with A/NP+A/M DNA and then exposed to HK/156 had antibodies reactive with HK/156 and cross-reactive with HK/483 and HK/485 viruses in HI, while control naïve mice had no detectable antibody (data not shown).

## Discussion

The most straightforward approach to vaccination against a newly emerging influenza subtype is use of inactivated virus or recombinant HA. However, if antigenically matched vaccines were not available in time or in sufficient quantity, other options would be important. Our study examines one of these.

DNA vaccination using genes for conserved antigens could have several advantages. The constructs could be available at any time. Plasmid production in bacteria is more consistent than growth of different viruses in eggs, and a cold chain might not be necessary for storage. To explore the usefulness of this approach, we studied the ability of NP+M DNA vaccines derived from A/PR/8 (H1N1) to protect against H5N1 challenge.

Vaccination with A/NP+A/M DNA readily induced antigen-specific antibody and T-cell responses, as shown previously (26,27). We investigated the potential for A/NP+A/M DNA vaccination to control infection by H5N1 viruses of modest (HK/486), intermediate (HK/156), and very high (HK/483) virulence phenotypes. Upon challenge with HK/486, a

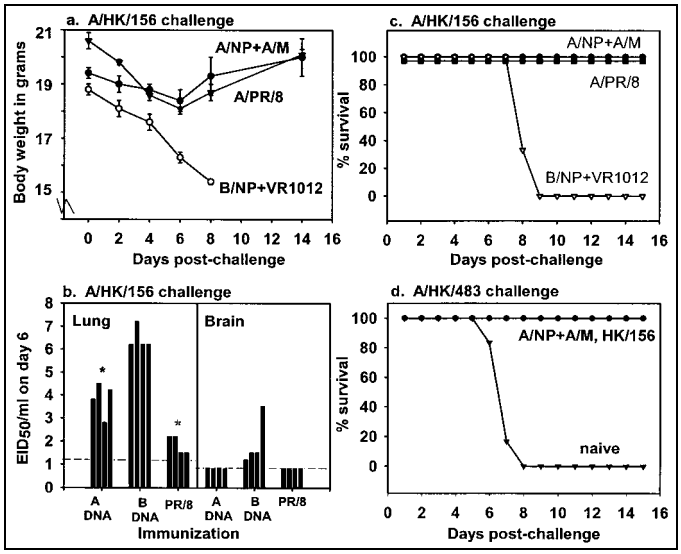


Figure 2. Mice immunized with influenza A nucleoprotein and matrix DNA (A/NP+A/M DNA) are protected against lethal A/Hong Kong/156/97 (HK/156) challenge. Mice were vaccinated as in Figure 1 with A/NP+A/M DNA, with influenza B nucleoprotein DNA (B/NP+blank DNA), or with 100 mouse infectious dose $(MID)_{50}$ of influenza A/Puerto Rico8/34 (A/PR/8) live virus. Sixteen days after the last dose of DNA, mice were challenged with 10,000 $MID_{50}$ of HK/156/97 intranasally. a) Monitoring of morbidity by body weight loss. b) Viral titers of lung and brain homogenates. Each bar represents the result for one mouse. Dashed lines indicate detection limits. Compared to the B/NP DNA controls, lung titers were significantly reduced in the A/NP+A/M DNA group (p=0.001, analysis of variation (ANOVA)) and the A/PR/8 group (p<0.001, ANOVA). c) Survival after challenge with HK/156. d) Survival after rechallenge with 100 $MID_{50}$ of HK/483 of mice primed with A/NP+A/M DNA and which had all survived the previous HK/156 infection.

strain that is not lethal in mice, lung titers were reduced approximately 17-fold. In previous work, even a 5- to 10-fold reduction in peak lung virus titers correlated with immunity protective against lethal challenge (14), so a 17-fold reduction and the accompanying difference in kinetics of viral clearance could alter biologic outcomes. In a test of its effectiveness, the vaccination provided benefit in the case of lethal challenge with HK/156, resulting in 100% survival and minimal morbidity as measured by weight loss, while unvaccinated controls demonstrated large weight losses and 100% death rates. After surviving HK/156 infection, the mice were resistant to lethal HK/483 challenge. Antibodies to HK/156 were demonstrated by HI to be present and cross-reactive with HK/483 virus before HK/483 challenge, which might account for the protection against HK/483. Of mice vaccinated only with A/NP+A/M DNA, half survived challenge with a dose of HK/483 lethal to all controls. While not statistically significant, the trend suggests some impact from immunization.

Regarding immune mechanisms of protection by A/NP+A/M DNA vaccination, candidates include CTL specific for NP (17) and antibodies to the N-terminal portion of M2 (18). Containing an infection with the kinetics of HK/483 may be difficult because it reaches near peak titers in as little as 24 hours. Only neutralizing antibody may be effective that early. Antigen presentation and reactivation of T-cell effectors take several days. However, when T cells specific for viral antigens are expanded substantially, they can reduce replication of highly lethal influenza viruses and clear infection more rapidly (41).

Comparing amino acid sequences in GenBank from viruses of five subtypes, NPs were $\geq$90% identical, with considerable conservation of known dominant CTL epitopes. M1 sequences were $\geq$94% conserved, while M2 sequences varied somewhat more. However, not all protective epitopes are known, and even single mutations can alter protective epitopes. Therefore, studies like the present one are necessary for establishing the range of virus strains against which a vaccine can work.

H5 viruses differ in virulence, and one cannot predict which strain might emerge in a future pandemic. With the threat of a pandemic and suboptimal existing vaccine candidates, new approaches to influenza vaccination should be considered. Our results suggest that DNA vaccination with conserved components has the potential to ameliorate disease caused by H5N1 viruses. The immunity induced by this mode of DNA vaccination does not completely prevent infection but passed the stringent test of protecting against lethal H5N1 challenge. Vaccines inducing neutralizing antibody could be administered subsequently to confer immunity against even the most virulent strains. In the absence of an antigenically matched HA-based vaccine, this approach might be useful as a first line of defense against a rapidly spreading influenza pandemic and should be further explored.

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# Antecedent Treatment with Different Antibiotic Agents as a Risk Factor for Vancomycin-Resistant *Enterococcus*

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We conducted a matched case-control study to compare the effect of antecedent treatment with various antibiotics on subsequent isolation of vancomycin-resistant *Enterococcus* (VRE); 880 in-patients; 233 VRE cases, and 647 matched controls were included. After being matched for hospital location, calendar time, and duration of hospitalization, the following variables predicted VRE positivity: main admitting diagnosis; a coexisting condition (e.g., diabetes mellitus, organ transplant, or hepatobiliary disease); and infection or colonization with methicillin-resistant *Staphylococcus aureus* or *Clostridium difficile* within the past year (independent of vancomycin treatment). After controlling for these variables, we examined the effect of various antibiotics. Intravenous treatment with third-generation cephalosporins, metronidazole, and fluoroquinolones was positively associated with VRE. In our institution, when we adjusted the data for temporo-spatial factors, patient characteristics, and hospital events, treatment with third-generation cephalosporins, metronidazole, and fluoroquinolones was identified as a risk factor for VRE. Vancomycin was not a risk factor for isolation of VRE.

First isolated in the late 1980s (1,2), vancomycin-resistant enterococci (VRE) have rapidly become established as important nosocomial pathogens in the United States. In some hospitals, VRE are responsible for >20% of enterococcal infections (3).

Given the complex genetic machinery required to confer vancomycin resistance, de novo emergence of resistance is unlikely in an individual patient (4). Thus, newly detected VRE may represent either acquisition of resistant organisms (or genes) or expansion of preexisting but undetected populations of VRE with which the patient is colonized (5). The likelihood of nosocomial VRE may vary with time and space, according to the endemicity of VRE in a specific location (i.e., colonization pressure) and to the duration of hospitalization (i.e., time at risk) (6,7). Indeed, initially most VRE isolates were recovered from patients in intensive-care units (ICUs); later VRE became more prevalent in patients on other wards (3). Certain coexisting conditions, e.g., malignancies, organ transplants, and chronic renal failure, were found to be associated with increased risk for VRE, as were exposure to contaminated equipment and proximity to a VRE carrier (8–16).

The effect of antecedent treatment with various antibiotic agents as a risk factor for nosocomial VRE has been explored in numerous studies, with conflicting results. Antimicrobial agents are believed to predispose to nosocomial VRE largely through effects on competing gastrointestinal microflora. Epidemiologic studies have identified therapy with vancomycin as a risk factor for VRE infection or colonization (8–19). A few studies have demonstrated an association between VRE and other antibiotic agents, including cephalosporins, quinolones, and metronidazole (12,14,18,19). However, no published study has directly compared multiple antibiotic agents while controlling for confounding.

Recently, we systematically reviewed published studies and provided evidence that questioned the relationship between vancomycin use and individual risk for nosocomial VRE colonization and infection (20). We suggested that the reported association might result from confounding as a result of selection of an inappropriate control group, lack of control for differences between cases and controls in duration of hospital stay, and publication bias. To conduct a study that examines multiple antibiotic agents simultaneously while controlling for confounding, a large number of VRE cases and controls are needed. Ideally designed, a study should be conducted in which serial cultures are collected prospectively to document the timing of change in patient status from VRE negative to VRE positive. However, such a study will be expensive and labor intensive. Only a few studies have been performed in which serial cultures were taken; these were conducted at high-incidence units and their small sample size made it difficult to control for multiple confounding (7,19,21). Thus, a retrospective study in which patients are included on the basis of clinical cultures was the only practical option.

Using this approach, we conducted a matched case-control study comparing the effect on VRE isolation of antecedent treatment with various antibiotics while controlling for temporo-spatial factors such as length of stay, hospital location, and calendar time, as well as patient characteristics.

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## Methods

The Beth Israel Deaconess Medical Center–West Campus is a 320-bed urban tertiary-care teaching hospital in Boston, Massachusetts. It has 24 ICU beds and approximately 12,000 patient admissions each year. The institutional antibiotic policy requires approval by an infectious disease consultant for the use of third-generation cephalosporins (other than ceftriaxone), antipseudomonal agents, and vancomycin (for more than one dose).

Data were collected from administrative, pharmacy, clinical, and laboratory computerized databases by using a relational database management system (Access, Microsoft Corp., Redmond, WA). The databases and methods of data collection have been described (22).

Enterococci were identified from clinical specimens submitted to the microbiology laboratory by using the Gram-Positive Identification Panel (Dade Behring Inc., Deerfield, IL). Enterococci were screened for vancomycin resistance by plating on brain heart infusion agar with 6 μg/mL vancomycin. Vancomycin resistance was confirmed by formal MIC testing with the microdilution broth method (MicroScan, Dade International Inc.). Isolates with vancomycin MICs ≥8 μg/mL were classified as VRE.

### Definitions and Study Design

The study was designed as a matched case-control study. All inpatients from whom VRE were first isolated from a clinical culture (either infected or colonized patients) in our hospital from October 1, 1993, through December 31, 1997, were enrolled as cases. Patients transferred from another institution and known to be VRE positive at that time were not included in this study. Patients and controls were matched on the basis of three variables: hospital ward, calendar time (within 7 days), and duration of hospital stay at the time of matching (up to 3 days' difference if no exact match was available). Up to three appropriately matched control-patients who were not VRE positive (i.e., patient was cultured and no VRE were isolated or the patient was never cultured) were randomly selected for each case. A list of all possible controls was created. Each was assigned a random number, and the three highest random numbers were chosen (without replacement). We looked for risk factors by examining demographics, admitting diagnosis, coexisting conditions (based on ICD-9 codes and electronic records), transfer from another institution, admission to an ICU and number of days in ICU, major surgical procedure, and infection with *Clostridium difficile* or methicillin-resistant *Staphylococcus aureus* (MRSA). After controlling for confounding by these variables, we compared, in detail, antecedent treatments with different antibiotic agents.

### Statistical Analysis

Statistics were run on Stata (Stata Corp., College Station, TX) software. A matched (conditional) logistic regression model was used. All variables other than antibiotic exposures were candidates for the model and were selected in a stepwise manner with an entry criterion of $p<0.2$ and a criterion to stay in the model of $p<0.05$. Variables that were not retained in the model by this procedure were then tested for confounding by adding them one at a time to the model and examining their effects on the β-coefficients. Variables that caused substantial confounding (change in β-coefficient of >10%) were included in the final model. After constructing the explanatory model, we examined the effect of treatment with each antibiotic by adding them to the model. The effects of antibiotic treatment were also examined by including them in the model and excluding possible collinear variables that were part of the explanatory model (e.g., vancomycin and infection or colonization with MRSA). In addition to examining statistical significance and confounding, we evaluated effect modification between variables by testing appropriate interaction terms for statistical significance. All statistical tests were two-tailed. A value of $p<0.05$ was considered significant.

## Results

During the 51-month study period, the incidence of VRE increased from 34 to 88 cases per 10,000 admissions. VRE were isolated in clinical cultures from 251 patients who fulfilled the study criteria (first isolation of VRE while hospitalized in our institution). The 251 diagnostic cultures were sent from 30 different nursing units. Twenty-eight percent of the case-patients were diagnosed during an ICU stay. No appropriate control patient could be matched for 18 cases. Thus, the study included 880 patients—233 cases and 647 matched control patients. The average age was 62 years (range 17–105), and 46% of the patients were female. Patients were hospitalized for an average of 8.1 days before entry into the study. The likelihood of being cultured (between admission and 2 days before matching) for cases and controls had similar distribution of the likelihood of being cultured (median 0, 0; 75th percentile 0, 1; and 90th percentile 21, 24 cultures for controls and cases, respectively).

The patients' characteristics with the unadjusted associated relative risks (odds ratios [OR]) for nosocomial VRE are shown in Table 1. Univariate matched analysis showed that case-patients were more likely than controls to be hospitalized for gastrointestinal and infectious conditions and less likely to be admitted for a cardiovascular condition. Case-patients were also more likely than controls to be solid organ transplant recipients and to have one of the following coexisting conditions: diabetes mellitus, renal disease, or hepatobiliary disease. Case-patients had higher chronic coexisting condition (Charlson) scores than controls and were less likely to have had major surgery during the index admission. Case-patients were also more likely than controls to have been infected (or colonized) within the past year with MRSA or *C. difficile*.

We developed a multivariate model to explain the likelihood of being VRE positive (Table 2). After being matched for hospital location, calendar time, and duration of hospitalization, the following variables predicted being VRE positive: 1) main admitting diagnosis; 2) coexisting conditions of diabetes

Table 1. Patient characteristics and matched univariate analysis for isolation of nosocomial vancomycin-resistant enterococci

| Variable | Cases (%) (233) | Control (%) (647) | Odds ratio | p value |
|---|---|---|---|---|
| Age[a] | 61.7 | 62.3 | .999 | 0.76 |
| Gender (female) | 114 (49) | 292 (45) | 1.2 | 0.34 |
| Orthopedic condition | 5 (6.4) | 53 (8.2) | R | R |
| **Main admitting diagnosis** | | | | |
| Cardiovascular condition | 49 (21) | 236 (36) | 0.37 | <0.001 |
| Endocrine disorder | 6 (2.6) | 13 (2) | 1.3 | 0.6 |
| Gastrointestinal disorder | 77 (33) | 160 (25) | 1.7 | 0.005 |
| Genitourinary disorder | 18 (7.7) | 35 (5.4) | 1.4 | 0.21 |
| Infectious disease | 26 (11) | 19 (3) | 3.7 | <0.001 |
| Hematologic disease | 5 (2.1) | 12 (1.9) | 0.83 | 0.7 |
| Neurologic disease | 18 (7.7) | 69 (10.7) | 0.72 | 0.3 |
| Pulmonary disease | 19 (8.1) | 49 (7.6) | 1.1 | 0.8 |
| **Coexisting conditions** | | | | |
| Cardiovascular disease | 160 (69) | 460 (71) | 0.85 | 0.36 |
| Lung disease | 32 (14) | 88 (14) | 1 | 0.95 |
| Diabetes mellitus | 127 (55) | 262 (40) | 1.9 | <0.001 |
| Organ transplant recipient | 39 (17) | 45 (7) | 2.9 | <0.001 |
| Renal disease | 57 (24) | 103 (16) | 1.6 | 0.013 |
| Cancer | 25 (11) | 93 (14) | .71 | 0.18 |
| AIDS | 2 (1) | 8 (1) | .75 | 0.7 |
| Hepatobiliary disease | 65 (28) | 97 (15) | 2.5 | <0.001 |
| Charlson comorbidity score[a] | 3.2 | 2.7 | 1.114 | 0.003 |
| Transfer from an institution | 84 (36) | 243 (37) | 0.9 | 0.56 |
| Surgery | 67 (29) | 211 (33) | 0.55 | 0.01 |
| Admission to ICU | 65 (28) | 169 (26) | .78 | 0.38 |
| **MRSA** | | | | |
| During current admission | 18 (8) | 16 (2.5) | 3.9 | 0.001 |
| In past year | 28 (12) | 26 (4) | 3.5 | <0.001 |
| **Clostridium difficile** | | | | |
| During current admission | 5 (2.1) | 10 (1.5) | 1.3 | 0.59 |
| In past year | 17 (7.3) | 20 (3.1) | 2.6 | 0.006 |

[a]Continuous variable.
R, reference group; MRSA, methicillin-resistant *Staphylococcus aureus*.

mellitus, organ transplant, or hepatobiliary disease; and 3) infection or colonization with MRSA or *C. difficile* within the past year. In the model adjusting for these variables, we examined the effect of being treated with each antibiotic (the adjusted effect). The unadjusted and adjusted effects of antecedent treatment with each agent are summarized in Table 3. Univariate (unadjusted) matched analysis disclosed that case-patients were more likely to have been treated with intravenous penicillins, third-generation cephalosporins, vancomycin, metronidazole, and quinolones (ciprofloxacin and ofloxacin). After we controlled for confounding, only intravenous treatment with cephalosporins, in particular third-generation cephalosporins, and with metronidazole was positively associated with VRE. Few patients were treated with oral antibiotics (<6%), and no significant association was found between enteral vancomycin (OR 1.2, p=0.83), metronidazole (OR 1.5, p=0.29), or clindamycin (OR 1.0, p=0.9) and VRE.

We examined the data to determine whether colinearity of vancomycin with MRSA or *C. difficile* infection explained the lack of significance of parenteral vancomycin in the adjusted analysis. A model that included vancomycin but excluded MRSA or *C. difficile* infection was constructed. In this model as well, treatment with vancomycin was not associated with VRE positivity (OR 1.3; p=0.8).

We also examined the effect of duration of treatment with each of the agents studied. The duration of treatment with vancomycin (OR 0.99; p=0.66), metronidazole (OR 1.03; p=0.18), and third-generation cephalosporins (OR 1.01, p=0.66) was not associated with VRE. In contrast, longer treatment with quinolones was associated with VRE both in the unadjusted analysis (OR 1.03; p=0.03) and in the multivariate model (OR 1.03; p=0.05).

In our final model (Table 3), the antibiotics included were third-generation cephalosporins (OR 2.9; p<0.001), intravenous metronidazole (OR 2.0; p=0.012), and long-term use of fluoroquinolones (OR 1.034; p=0.027).

## Discussion

VRE is a major emerging pathogen that has spread rapidly since these organisms were first detected approximately a decade ago (23). Antibiotics, particularly vancomycin, have been ascribed a crucial role in the dissemination of VRE; yet, many publications addressing this subject had small sample sizes or control groups, focused on a limited number of antimicrobial agents, or did not completely control for confounding factors. Thus, the true relationship between vancomycin and VRE and the relative importance of antimicrobial agents other than vancomycin have remained unclear.

In this study, the largest reported to date on VRE, we systematically compared the major classes of antibiotics used in the hospital setting for their association with VRE infection. The effects of duration of treatment and route of administration (i.e., oral and parenteral) were also examined. We controlled for temporo-spatial factors, correlates of transmission, and duration of risk by matching case-patients and controls for hospital location, calendar time, and duration of hospital stay until diagnosis. We considered length of stay to be particularly important because it represents the duration of the at-risk period for both exposure to antibiotics and acquisition of VRE and, in addition, is a correlate of severity of illness. Multivariable models were used to address other potential confounding factors such as surgical procedures, coexisting conditions, and reason for hospitalization.

Our major findings were 1) vancomycin was not associated with VRE positivity, a finding consistent with the results

Table 2. Multivariable explanatory model for having vancomycin-resistant enterococci–positive case

| Variable | Odds ratio (95% CI) | p value |
|---|---|---|
| Main admitting disorder | 0.44 (0.28 to 0.68) | <0.001 |
| Cardiovascular | 2.9 (1.5 to 5.7) | 0.002 |
| Infectious | | |
| Coexisting conditions | | |
| Diabetes mellitus | 2.1 (1.5 to 3.1) | <0.001 |
| Transplant recipient | 2.6 (1.6 to 4.5) | <0.001 |
| Hepatobiliary disease | 2.9 (1.8 to 4.6) | <0.001 |
| MRSA (in past yr) | 3.5 (1.8 to 6.9) | <0.001 |
| *Clostridium difficile* (in past yr) | 2.0 (0.97 to 4.3) | 0.06 |

CI, confidence interval; MRSA, methicillin-resistant *Staphylococcus aureus*.

of the meta-analysis on this subject (20); 2) third-generation cephalosporins and parenteral metronidazole were highly significant independent risk factors for VRE; and 3) only fluoroquinolones exhibited a statistically significant linear relationship between intensity (duration) of exposure and risk for VRE. In contrast, for metronidazole and third-generation cephalosporins, a threshold-type (all or none) effect was observed. The risk for VRE in patients treated with these agents was increased regardless of duration of therapy.

The small effect of parenteral vancomycin in the unadjusted analysis was completely erased after the data were controlled for confounding by patient characteristics and treatment with other antibiotics, mainly treatment with third-generation cephalosporins and metronidazole. Thus, individual patients who received vancomycin did not appear to be at any increased risk for VRE infection. We believe that the lack of effect of vancomycin on VRE found in this study, a finding that contradicts the results of many earlier studies, relates to our compliance with adequate epidemiologic principles in study design and analysis. These principles include controlling for length of stay, choosing the control group from the source population, matching for endemicity by matching on time and location, and adjusting for other antibiotic exposures. Most early studies that identified vancomycin as a strong risk factor for VRE failed to account for these principles (24,25).

These data do not dispute the role of glycopeptide use in promoting the emergence of glycopeptide resistance; this role may be related to glycopeptides' effect on the possibility of a positive patient's becoming a transmitter, rather than on increasing the risk of the susceptible person's becoming colonized (26). Indeed, our study was aimed at the individual level and not at the group level. A recent study performed at the group level demonstrated that ICUs in which vancomycin is heavily used have higher rates of VRE (27). We believe that the discordant results between individual level and group level analysis (28) and the effect of glycopeptides on the possibility of transmission among the already colonized patients deserve further study. The results of our analysis, as well as results of other studies (6,7,20), call into question whether restricting vancomycin will lower VRE incidence.

The effect of third-generation cephalosporins on risk for VRE is likely due to their activity against nonenterococcal aerobic enteric flora, leading to decrease in resistance colonization, allowing colonization with VRE. Similarly, suppression of gastrointestinal anaerobic flora is the presumed mechanism for the association between metronidazole and VRE. This activity and suppression do not explain the lack of effect of other agents with similar or even broader spectra of activity such as clindamycin, β-lactamase–inhibitor combinations, and imipenem. Other researchers have suggested that the combina-

Table 3. The effect of antibiotic treatment as risk factor for vancomycin-resistant enterococci

| | Cases (%) | Control (%) | Unadjusted effect | | Adjusted for explanatory model[a] | | Adjusted for model and other antibiotics[a] | |
|---|---|---|---|---|---|---|---|---|
| Antibiotic agent | (233) | (647) | OR | p value | OR (95% CI) | p value | OR (95% CI) | p value |
| Penicillins | 67 (29) | 134 (21) | 1.5 | 0.04 | .99 (.63 to 1.6) | 0.97 | 1.0 (.64 to 1.7) | 0.86 |
| β-lactam-inhibitor combination | 49 (21) | 98 (15) | 1.5 | 0.07 | .94 (.6 to 1.5) | 0.78 | | |
| Cephalosporins | 104 (45) | 248 (38) | 1.2 | 0.28 | 1.5 (1.0 to 2.4) | 0.048 | | |
| Third generation | 69 (30) | 97 (15) | 2.6 | <0.001 | 2.8 (1.7 to 4.5) | <0.001 | 2.8 (1.7 to 4.8) | <0.001 |
| Vancomycin (p.o.) | 4 (1.7) | 7 (1.1) | 1.2 | 083 | 1.0 (.25 to 4.2) | 0.96 | | |
| Vancomycin (i.v.) | 67 (29) | 121 (19) | 1.7 | 0.016 | 1.4 (.89 to 2.3) | 0.19 | .99 (.57 to 1.7) | 0.98 |
| Metronidazole (p.o.) | 13 (5.6) | 23 (3.6) | 1.5 | 0.29 | 1.0 (.42 to 2.5) | 0.97 | | |
| Metronidazole (i.v.) | 47 (20) | 57 (9) | 2.5 | <0.001 | 2.3 (1.3 to 3.9) | 0.003 | 2.1 (1.2 to 3.7) | 0.008 |
| Clindamycin | 20 (8.6) | 51 (7.9) | 1 | 0.9 | 1.5 (.76 to 2.8) | 0.26 | 1.1 (.55 to 2.3) | 0.76 |
| Quinolone[b] | 48 (21) | 68 (11) | 2 | 0.005 | 1.6 (.94 to 2.6) | 0.086 | 1.5 (.85 to 2.6)[b] | 0.17[b] |
| Imipenem | 19 (8.2) | 27 (4.2) | 1.7 | 0.12 | 1.3 (.61 to 2.9) | 0.47 | 1.2 (.52 to 2.8) | 0.66 |

[a]Adjusted for the explanatory model detailed in Table 2.
[b]When included as a continuous variable (number of days of treatment with quinolone) OR=1.03, p=0.05.
OR, odds ratio; p.o., orally; i.v., intravenously.

tion of enteric concentration of the antimicrobial agent and its spectrum of activity against competing microflora determines its likelihood to be a risk factor (Rice LB, unpub. data). In our study, the positive association between duration of quinolone treatment and VRE had borderline statistical significance and a small increased risk per day of treatment. This observation, which requires further validation, may have important clinical importance for patients treated for long durations.

We also found that patients who were VRE positive were more likely to have been infected or colonized with MRSA or *C. difficile* in the past year. This association has been previously described (29) and, in our data, was independent of vancomycin treatment. This relationship likely reflects shared mechanisms of acquisition for these nosocomial pathogens and a common association with severity of illness.

Our study has certain limitations. We assumed that time of VRE positivity was similar to time of acquisition for cases. This assumption is likely incorrect but is the best possible estimate in this type of study. Studies based on serial surveillance cultures may yield a more accurate estimate of time of acquisition but cannot reach an adequate sample size to perform statistical analysis controlling for confounding. If we had performed serial cultures twice a week on all our source population, we would have processed >100,000 surveillance cultures. Indeed, almost all previous studies on this subject had a similar assumption. Control patients were representative of the hospital-based population but were not screened to exclude undetected VRE colonization. However, it is unlikely that misclassification bias could simultaneously account for the substantial effect observed with certain antibiotics and lack of effect observed with others. Moreover, the results of a meta-analysis also suggest that the magnitude of association between vancomycin treatment and VRE was independent of the method of VRE detection, i.e., clinical or surveillance cultures (20). Another caveat is that the results of this study apply to individual risk for VRE. Antibiotics may have differential effects on the quantity of VRE excreted from already colonized persons, as suggested both by animal models and human data (30–32). Thus, the effects of antibiotics on ecologic risk, e.g., transmission of VRE to other patients, may differ from their effects on individual risk (28). Finally, the power of this study to examine the effects of oral antibiotics was limited because of the small number of patients treated with these agents. Along the same lines, because of their limited use, these agents are unlikely to play a major role in the epidemiology of VRE within hospitals.

We conclude that patients treated with third-generation cephalosporins, metronidazole, or quinolones for an extended duration appear to be at significantly higher risk for VRE. Antecedent treatment with vancomycin is not a risk factor for VRE infection or colonization. Further studies to examine the routes of transmission of VRE and the ecologic role of antibiotics are needed.

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# Genetic Homogeneity of Measles Viruses Associated with a Measles Outbreak, São Paulo, Brazil, 1997

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During a resurgence of measles in São Paulo, Brazil, in 1997, >40,000 cases (peak incidence rate of 246/100,000 inhabitants) and 42 measles-related deaths were reported. Reverse transcriptase-polymerase chain reaction and nucleotide sequencing were used to analyze specimens from patients who had typical clinical measles infection during this outbreak and from six patients who had had measles in 1995 and 1996. Although wild-type measles viruses (genotypes D5 and D6) were present in São Paulo before this resurgence, we detected only D6 viruses. The genotype D6 viruses isolated during this outbreak had identical sequences to genotype D6 viruses isolated in other parts of Brazil and South America in 1997 and 1998, suggesting that a single chain of transmission was responsible. We also identified genotype A viruses in two vaccine-associated cases from 1995 and 1996. Our findings extend the knowledge of the circulation patterns of measles virus in South America, contributing to measles control efforts in the Americas.

The Pan American Health Organization (PAHO) reported record low numbers of measles cases for 2000–2001, reflecting the success of measles control programs in the Western Hemisphere (1). In the most populous country in the region, the United States, indigenous transmission of measles virus has been eliminated since 1993, and only 3 of 41 countries in the region reported indigenous measles transmission during 2001 (1,2). Despite this success, measles remains an endemic disease in many areas of the world. The World Health Organization (WHO) estimates that approximately 45 million cases of measles and >800,000 measles-related deaths continue to occur annually (3,4). Sporadic measles outbreaks still occur in both developed and undeveloped countries that have failed to maintain adequate immunization levels. These outbreaks, such as the one that occurred in São Paulo, Brazil, in 1997, provide an opportunity to study the virologic and epidemiologic aspects of measles transmission in vaccinated populations.

Before the introduction of vaccine, measles was a substantial public health problem in Brazil, representing a major cause of death among young children. Even after the introduction of vaccine, measles epidemics continued to occur in Brazil. For example, the epidemics of 1984 and 1986 had incidence rates of 63/100,000 and 97/100,000, respectively. PAHO launched an aggressive measles control program for the Americas in 1991 that included one-time national catch-up campaigns for all children, routine "keep-up" vaccination of infants at 1 year of age, and follow-up campaigns at 3- to 5-year intervals. In Brazil, this strategy resulted in a 93% reduction in the incidence of measles in Brazil from 1991 through 1996. For the 4-year period 1993–1996, <1,000 cases were reported annually. However, during the resurgence of measles in 1997 in São Paulo, 42,055 confirmed cases of measles (23,907 laboratory confirmed), were reported, with a peak incidence rate of 246/100,000 inhabitants. Most of the cases were in adults living in metropolitan São Paulo. Forty-two deaths caused by measles were reported during this epidemic (5–7).

Virologic surveillance can help to identify the transmission pathways of measles virus and is an important component of measles surveillance activities (8–10). Sequence analysis of measles viruses has shown that distinct lineages of wild-type viruses exist and co-circulate, and WHO currently recognizes 20 measles genotypes (11–13). The epidemiologic aspects of the measles outbreak in São Paulo have been described (5–7). The purpose of this report is to present the genetic characteristics of the measles viruses responsible for the resurgence of measles in São Paulo, Brazil, during 1997. This outbreak provided an opportunity to study the transmission of viruses in an area that had previously had good measles control but then experienced a rapid resurgence in cases. Our study showed that a single genotype of virus, D6, was responsible for the outbreak and that viruses from this chain of transmission subsequently spread to other countries in South America.

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## Materials and Methods

### Specimens

Samples from patients with laboratory-confirmed measles were analyzed (Table). Six of the samples were serum specimens; the rest were heparinized, whole-blood specimens. Peripheral blood mononuclear cells (PBMC) were purified from whole blood by using Ficoll-Hypaque gradient centrifugation. The PBMC were washed three times with phosphate-buffered saline before being resuspended in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS). Aliquots of PBMC were cryopreserved by using standard procedures (14).

### Virus Isolation

B95a cells (15) were injected with 0.2 mL of the clinical samples and maintained in DMEM, 0.2 mM L-glutamine/100 mL and gentamicin 40 mg/mL, in 2% FCS at 37°C. Samples with no apparent cytopathic effect (CPE) after 3 passages were discarded as negative for virus isolation. If CPE was observed, the cells were harvested by centrifugation when CPE was maximal, and RNA was extracted from the cell pellet. Five measles viruses were isolated from PBMC collected during the epidemic.

### RNA Extraction, Polymerase Chain Reaction, and Sequencing

RNA was extracted from serum, PBMC, or infected cell pellets by the guanidinium acid-phenol method (16). cDNA corresponding to the nucleotides coding for the carboxyl terminus of the nucleoprotein (N) or the full-length open reading frame for the hemagglutinin (H) gene were synthesized by using avian myeloblastosis virus reverse transcriptase (RT) and amplified by polymerase chain reaction (PCR), and the sequences of the PCR products were obtained as described previously (17,18). Sequence data were analyzed by using version 10.1 of the Genetics Computer Group Sequence Analysis Software Package (Accelrys Inc., Madison, WI) (18) and phylogenetic analyses were performed with PAUP version 4.0 (19). A reference N gene sequence was deposited in GenBank under accession number AF495863.

## Results

We obtained genetic information from specimens that were collected in São Paulo from March 1995 to October 1997 (Table). During 1995 and 1996, relatively few measles cases were reported in São Paulo; six acute-phase serum samples were obtained from confirmed, sporadic cases that occurred as early as 2 years before the outbreak. In 1997, a resurgence of measles occurred in São Paulo, beginning in the fall and peaking in late winter (Figure 1). Twenty-three samples were obtained from cases that occurred during the first 42 weeks of 1997; 11 of these samples were obtained in August 1997 during the peak of the outbreak (Table). We obtained most of the outbreak specimens from young adults, which is consistent with the observation that most of the measles cases occurred in unvaccinated, young adults (6). Measles-specific immunoglobulin (Ig) M responses were detected in all the patients listed in the Table, except one. The serum sample from case number 802 was negative, probably because it was obtained <3 days after rash onset. However, this case was confirmed by a positive virus isolation (Table).

Virus isolation was not attempted for the serum samples, but five virus isolates were obtained from the PBMC specimens (Table) obtained during 1997. The sequences coding for the carboxyl terminus of the nucleoprotein (456 nucleotides) of these isolates were compared with the sequences of the WHO reference strains (12). The results indicated that these Brazilian viruses all had identical N gene sequences and were members of genotype D6 (Figure 2). The N gene sequence of MVi/SãoPaulo.BRA/42.97/35175 has been deposited in GenBank (accession no. AF495863) as a reference sequence for this outbreak. The complete sequence of the H gene was also obtained for MVi/SãoPaulo.BRA/42.97/35175. Comparison of this H gene sequence with the sequences of the H genes from the WHO reference strains confirmed the placement of these viruses in genotype D6 (data not shown).

For the rest of the specimens from 1997, nucleotide sequences were obtained from PCR products that were amplified directly from RNA extracted from the PBMC specimens. In most cases, we had to perform a nested PCR reaction to get sufficient material for sequencing. All specimens obtained in 1997, either just before or during the peak of the outbreak, were placed in genotype D6. Most of the N gene sequences obtained from these specimens were identical to each other and to the N gene sequences obtained from the five isolates from 1997. Only three of the specimens had sequences that differed from the majority sequence by a single nucleotide.

We were also able to amplify measles-specific PCR products from the serum samples obtained from sporadic measles cases that occurred in 1995 and 1996. Two of these specimens had nucleotide sequences in genotype A, which contains all vaccine viruses. Both specimens were obtained from 9-month-old infants who had received their measles vaccination 12–13 days before collection of the serum specimen. One of the samples obtained in 1995 had an N gene sequence in genotype D5, while the other three serum samples had sequences in genotype D6 identical to the D6 sequences obtained during the outbreak. These data showed that D6 viruses and wild-type viruses from genotypes other than D6 were present in São Paulo as early as 2 years before the peak of the outbreak in 1997.

We compared the N gene sequences from the genotype D6 viruses in the São Paulo cases with N gene sequences from genotype D6 viruses isolated in other parts of South America (20–24) and the world (Figure 2). For simplicity, three representative sequences from the South American viruses are shown in the phylogenetic tree. The sequences from the São Paulo outbreak were identical to those obtained from viruses isolated in Brazil (i.e.,Valenca, Rio de Janeiro, Belo Horizonte,

Table. Summary of 41 measles specimens analyzed, São Paulo, Brazil, March 1995–October 1997

| Name[a] | ID[b] | Age | Date of specimen collection | Vaccination status[c] | Genotype[a] |
|---|---|---|---|---|---|
| MVs/São Paulo.BRA/10.95 | 205* | 9 m | 03/09/95 | 02/24/95 | A |
| MVs/São Paulo.BRA/35.95/2 | 258* | 9 m | 08/30/95 | Yes | D5 |
| MVs/São Paulo.BRA/35.95/1 | 2793* | 9 m | 08/29/95 | 08/19/95 | D6 |
| MVs/São Paulo.BRA/3.96 | 141* | 9 m | 01/15/96 | 01/03/96 | A |
| MVs/São Paulo.BRA/5.96 | 431* | 9 m | 02/26/96 | 02/08/96 | D6 |
| MVs/São Paulo.BRA/27.96 | 1490* | 9 m | 07/03/96 | 06/15/96 | D6 |
| MVs/São Paulo.BRA/4.27 | 106 | 25 yr | 01/20/97 | No | D6 |
| MVs/São Paulo.BRA/6.97 | 267 | 20 yr | 02/04/97 | Yes | D6 |
| MVs/São Paulo.BRA/9.97 | 503 | 30 yr | 03/01/97 | Unknown | D6 |
| MVs/São Paulo.BRA/10.97 | 561 | 24 yr | 03/06/97 | Unknown | D6 |
| MVs/São Paulo.BRA/11.97 | 661 | 20 yr | 03/11/97 | No | D6 |
| MVs/São Paulo.BRA/12.97/3 | 760 | 27 yr | 03/18/97 | No | D6 |
| MVs/São Paulo.BRA/12.97/1 | 764 | 23 yr | 03/17/97 | Unknown | D6 |
| MVs/São Paulo.BRA/12.97/2 | 765 | 31 yr | 03/17/97 | No | D6 |
| MVi/São Paulo.BRA/12.97/4 | 802 | 29 yr | 03/18/97 | No | D6 |
| MVs/São Paulo.BRA/12.97/5 | 862 | 26 yr | 03/18/97 | Unknown | D6 |
| MVs/São Paulo.BRA/14.97/2 | 1045 | 6 m | 04/02/97 | No | D6 |
| MVs/São Paulo.BRA/14.97/3 | 1048 | 27 yr | 04/02/97 | No | D6 |
| MVs/São Paulo.BRA/14.97/1 | 1084 | 3 m | 04/01/97 | No | D6 |
| MVs/São Paulo.BRA/15.97/1 | 1225 | 4 m | 04/07/97 | No | D6 |
| MVi/São Paulo.BRA/14.97/4 | 1139 | 22 yr | 04/03/97 | No | D6 |
| MVs/São Paulo.BRA/15.97/2 | 1202 | 2 yr | 04/10/97 | Unknown | D6 |
| MVs/São Paulo.BRA/17.97/1 | 1398 | 15 yr | 04/23/97 | No | D6 |
| MVi/São Paulo.BRA/17.97/3 | 1494 | 33 yr | 04/27/97 | No | D6 |
| MVs/São Paulo.BRA/17.97/2 | 8954 | 3 yr | 04/25/97 | Yes | D6 |
| MVs/São Paulo.BRA/32.97 | 9462 | 20 yr | 08/21/97 | Unknown | D6 |
| MVi/São Paulo.BRA/33.97/1 | 9463 | 25 yr | 08/12/97 | No | D6 |
| MVs/São Paulo.BRA/33.97/2 | 9778 | 24 yr | 08/13/97 | Unknown | D6 |
| MVs/São Paulo.BRA/30.97 | 9786 | - | 07/22/97 | Yes | D6 |
| MVs/São Paulo.BRA/34.97/4 | 10085 | 3 m | 08/21/97 | No | D6 |
| MVs/São Paulo.BRA/33.97/5 | 10171 | 30 yr | 08/15/97 | No | D6 |
| MVs/São Paulo.BRA/33.97/6 | 10172 | 26 yr | 08/15/97 | Unknown | D6 |
| MVs/São Paulo.BRA/33.97/3 | 10173 | 5 m | 08/14/97 | No | D6 |
| MVs/São Paulo.BRA/33.97/4 | 10175 | 24 yr | 08/14/97 | Unknown | D6 |
| MVs/São Paulo.BRA/33.97/8 | 10179 | 28 yr | 08/16/97 | Unknown | D6 |
| MVs/São Paulo.BRA/33.97/9 | 10181 | 22 yr | 08/17/97 | Yes | D6 |
| MVs/São Paulo.BRA/34.97/1 | 10242 | 25 yr | 08/19/97 | No | D6 |
| MVs/São Paulo.BRA/33.97/7 | 10660 | 20 yr | 08/15/97 | Yes | D6 |
| MVs/São Paulo.BRA/34.97/2 | 10661 | 3 yr | 08/19/97 | No | D6 |
| MVs/São Paulo.BRA/34.97/3 | 10705 | 31 yr | 08/21/97 | Unknown | D6 |
| MVi/São Paulo.BRA/42.97 | 35175 | 4 m | 10/14/97 | No | D6 |

[a]Sequence name and genotype designation based on nomenclature established by World Health Organization (11).
[b]ID, specimen identification number. All samples were from purified peripheral blood mononuclear lymphocytes except for serum samples, designated by an asterisk.
[c]Date of last measles vaccination, if known.

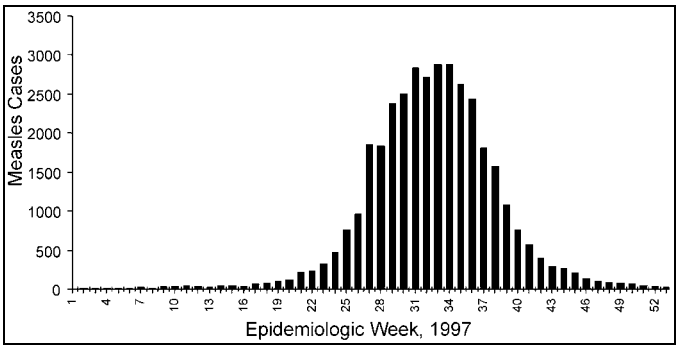


Figure 1. Number of measles cases, by week, São Paulo, Brazil, 1997.

Angra do Reis, and Curitiba) (20,21), Argentina (22,23), Uruguay (24), and Bolivia in 1997 and 1998. This homogenous group also included two viruses that were imported into the United States from Brazil in 1997 (25). The sequences from genotype D6 viruses imported into the United States (26, unpub. data) from European sources and genotype D6 viruses isolated in Europe (27,28) were slightly different from the South American D6 group. We found that the sequences from the South American viruses were most closely related to a genotype D6 virus isolated in Luxembourg in 1997, although no foreign source was identified for the outbreak in Brazil. Overall, the N genes sequences of D6 viruses within the South American group differed by no more than 0.4% but differed from other genotype D6 sequences by as much as 1.4%.

## Discussion

This report presents the virologic surveillance data from the measles outbreak that occurred in São Paulo, Brazil, during 1997. Sequence data from the specimens obtained during the outbreak indicated the presence of measles viruses belonging to genotype D6, which is one of the prevalent genotypes of measles virus in Europe. Frequently associated with imported measles cases from European countries (8,26), D6 viruses have been isolated in the United Kingdom, Spain, Germany, Russia, Poland, Denmark, and Luxembourg (27–31). During 1997–2000, these genotype D6 viruses were imported into the United States from Italy, Greece, Ukraine, Croatia, Cyprus, and the United Kingdom, and from the large measles outbreak that occurred in São Paulo (Rota, unpub. data). However, because neither conventional case nor virologic surveillance was conducted in Brazil before the start of the measles control program in 1991, it is not possible to determine if the D6 viruses were recently introduced into Brazil by importation or if they represent continued circulation of viruses indigenous to Brazil. Our data show that D6 viruses indistinguishable from the D6 viruses isolated during the São Paulo outbreak were responsible for sporadic measles cases in São Paulo for at least 2 years before the outbreak. In a large urban area such as São Paulo, the virus will likely be continually present either from low-level circulation or frequent importation. In late 1997, a change occurred in the epidemiologic circumstances, resulting in a large outbreak of the measles virus. Factors that contributed to the outbreak included the failure to conduct a timely follow-up vaccination campaign in 1995 and the low level of vaccination coverage among infants achieved by routine health services. Additionally, a large number of unvaccinated young adults who had escaped measles infection had moved to São Paulo from rural areas. The high population density in São Paulo also facilitated rapid spread of the virus (7).

Laboratory-based surveillance for measles was established in Brazil in the early 1990s. Although virologic surveillance was certainly incomplete, some information is available about measles genotypes present in Brazil before the 1997 outbreak in São Paulo. Genotype D5 was detected in a sporadic case in São Paulo in 1995 and in sporadic cases that occurred in the state of Bahia in 1996 (21). Genotype D5 viruses that circulate in Japan have been associated with importation of viruses from Japan into other countries (26,32,33). The source of the genotype D5 viruses detected in São Paulo and Bahia was not identified. Genotype C2 viruses were detected in a small outbreak in the state of Santa Catarina in 1996 (21). C2, another indigenous genotype in Europe, is often associated with imported cases in other parts of the world (27–31).

Fever and rash occur in approximately 5% of measles vaccine recipients (34). In areas where wild-type virus is circulating, vaccine reactions may be classified as measles cases. Distinguishing a vaccine reaction from a measles case is not possible with the currently available serologic methods. Only the genetic analysis of virus isolates can confirm the presence of either a vaccine or wild-type virus. In this study, two vac-

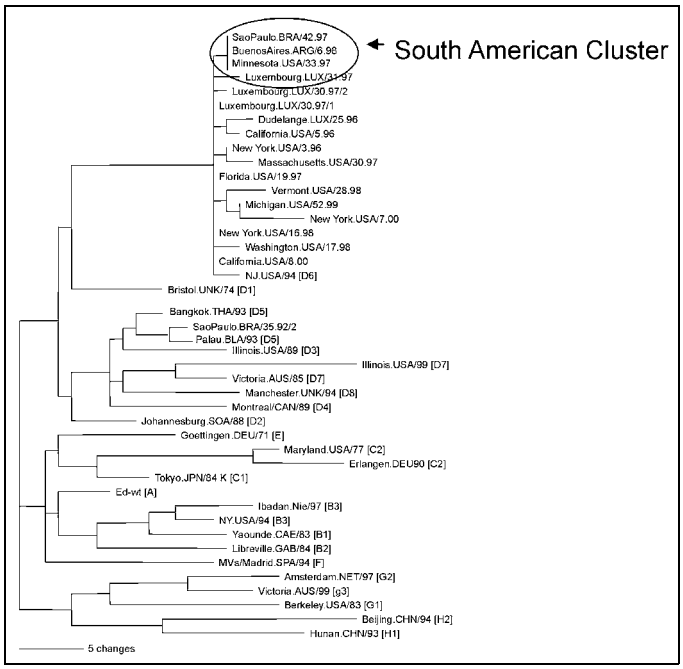


Figure 2. Phylogenetic relationships of the N gene sequences from genotype D6 viruses from Brazilian, South American, and European sources. Tree shows representative genotype D6 sequences from Brazil, Argentina, Uruguay, and Bolivia, and viruses imported into the United States from Brazil (South American cluster) compared with genotype D6 sequences of viruses isolated in Europe or imported into the United States from European sources. Also shown are the sequences of the World Health Organization reference strains for each genotype.

cine viruses were detected in persons who had received vaccine 12 and 13 days before collection of specimens. In vaccine reactions, rash and fever typically occur 7 to 12 days after vaccination; the virus can be detected in circulating lymphocytes even after the rash and fever have resolved.

The situation in Brazil is now very similar to that in the United States in the early 1990s. From 1989 to 1991, the United States experienced a resurgence of measles, with >50,000 reported cases. Virologic surveillance suggested that virus from a single genotype, D3, had seeded the entire country. As was the case with the D6 viruses from South America, the D3 viruses from many different areas in the United States had nearly identical sequences (8). Therefore, we conclude that when measles outbreaks occur in areas that have good measles control, genetic analysis of viruses will confirm the presence of a single chain of transmission. In contrast, in areas where measles control programs are less advanced, genetic studies have identified multiple chains of transmission within a genotype (17).

Initiation of a two-dose vaccination schedule, along with the success of the PAHO strategy in the Americas, resulted in historically low numbers of cases in the United States in the years following the resurgence there; both standard epidemiologic surveillance and molecular epidemiologic surveillance have documented the sustained interruption of transmission of the genotype D3 viruses (1,2,8,35–37). Similarly, the pattern of viral genotypes found in Brazil and other parts of South and Central America will help to assess the success of the PAHO measles control programs. Genotype D6 viruses were circulating in Haiti and the Dominican Republic during 2001 but have not been isolated from ongoing chains of transmission in any other countries in the region (36). During 2002, virologic surveillance showed that measles cases imported into El Salvador from Europe were genotype D7 (38). As the number of measles cases continues to decrease in the Americas, increasing virologic surveillance activities will be important. This virologic surveillance will document the interruption of indigenous transmission of the genotype D6 viruses in Brazil and in other parts of South America.

Dr. Oliviera is a research scientist at the Adolfo Lutz Institute, São Paulo, Brazil. Her research interests include diagnosis of exantematic viral diseases and molecular epidemiology of exantematic viral diseases, including measles, rubella, parvovirus B19 and herpevirus 6.

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# Serologic Evidence of H1 Swine Influenza Virus Infection in Swine Farm Residents and Employees

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We evaluated seropositivity to swine and human H1 influenza viruses in 74 swine farm owners, employees, their family members, and veterinarians in rural south-central Wisconsin, compared with 114 urban Milwaukee, Wisconsin, residents. The number of swine farm participants with positive serum hemagglutination-inhibition (HI) antibody titers $\geq$40 to swine influenza viruses (17/74) was significantly higher ($p<0.001$) than the number of seropositive urban control samples (1/114). The geometric mean serum HI antibody titers to swine influenza viruses were also significantly higher ($p<0.001$) among the farm participants. Swine virus seropositivity was significantly ($p<0.05$) associated with being a farm owner or a farm family member, living on a farm, or entering the swine barn $\geq$4 days/week. Because pigs can play a role in generating genetically novel influenza viruses, swine farmers may represent an important sentinel population to evaluate the emergence of new pandemic influenza viruses.

Infections with influenza viruses that circulate within the human population are a common and important cause of respiratory disease in people and result in an average of approximately 20,000 deaths and 114,000 hospitalizations per year in the United States alone (1–3). Influenza A viruses also infect animals of a wide variety of other species. In particular, influenza is a common and economically important cause of respiratory disease in pigs (4,5); subclinically infected wild waterfowl provide a global reservoir of influenza A viruses of all 15 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes (6,7).

The occurrence of H5N1 and H9N2 virus infections among people in Asia in 1997–1999 (8,9) highlighted the potential for avian influenza viruses to cross species barriers to infect humans, but direct avian-to-human transmission of influenza viruses is a rare event. In contrast, the species barrier for transmission of influenza viruses between people and pigs appears to be less stringent, and influenza virus infections in pigs pose important public health concerns at two levels. First, because respiratory tract epithelial cells in pigs contain the sialic acid receptors preferred by both avian ($\alpha$2,3-N-acetylneuraminic acid-galactose) and human ($\alpha$2,6-N-acetylneuraminic acid-galactose) influenza viruses (10), pigs are postulated to serve as the "mixing vessel" hosts in which reassortment between avian and human viruses can generate genetically novel viruses with pandemic potential (7,11,12). Reassortment between human and avian influenza viruses produced the 1957 and 1968 pandemic viruses (7). More recently, human-avian reassortant viruses have been isolated from pigs in Europe and, thereafter, from children in the Netherlands (13,14).

Zoonotic infections of humans with swine influenza viruses, first confirmed by isolation of swine influenza viruses from both pigs and their caretaker on a farm in southern Wisconsin in November 1976 (4), have been diagnosed in the United States, Europe, New Zealand, and Asia (15). However, the total number of zoonotic infections that have been described is relatively small compared to the number of people worldwide involved directly or indirectly in swine farming. Swine farm workers are likely to be routinely exposed to and infected with swine influenza viruses, but only a small percentage of those zoonotic infections are documented. Zoonotic infections may be recognized if information regarding contact with sick pigs is specifically communicated to physicians, if a patient is hospitalized or dies, or if virus isolation is pursued and yields a virus that is antigenically atypical. In most cases, however, swine influenza virus infections in people may not be clinically distinguishable from routine human influenza virus infections. We developed this study to serologically assess the relative level of exposure to classical H1 swine influenza viruses among people involved in swine farming.

## Methods

### Study Population and Design

Names and contact information for swine farmers living in rural areas of south-central Wisconsin were provided by area swine veterinarians. We contacted these farmers initially by telephone and then, if they were interested in participating, one of the study directors met with them to explain the project's

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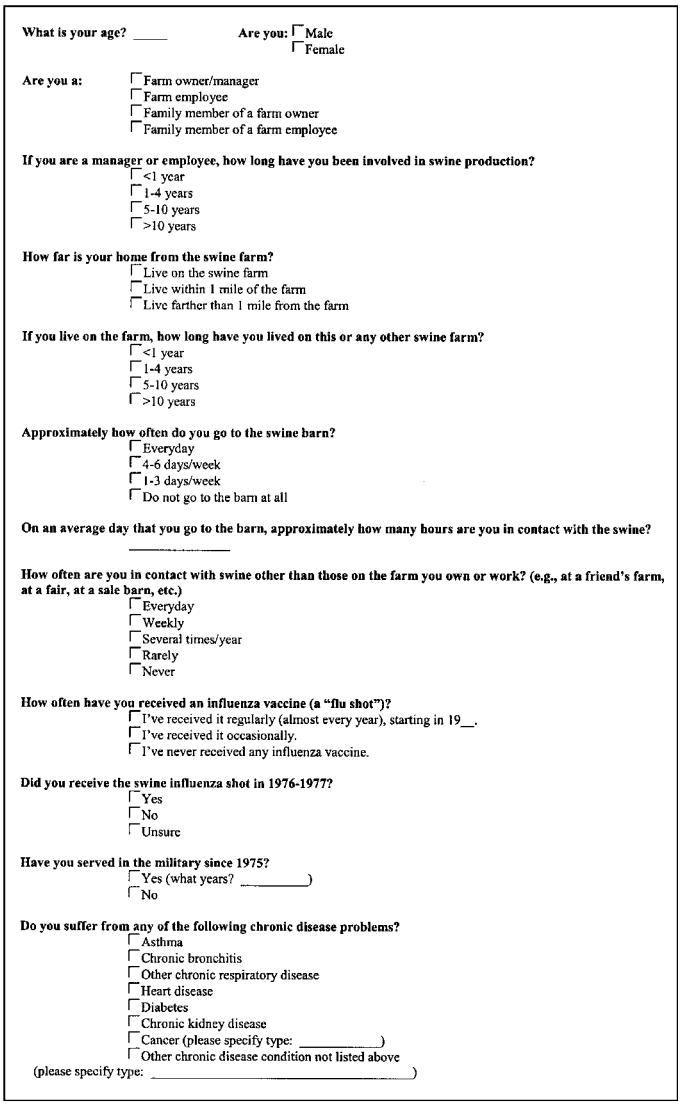
objectives and procedures. To take part in the study, persons were required to allow home health nurses to collect two blood samples for influenza virus serologic testing and to complete a questionnaire regarding their general health and the nature of their contact with pigs. Participation was also extended to other employees on the farm, spouses and children >7 years of age, and farm veterinarians. A total of 79 participants were initially enrolled, including 76 persons from 22 farms, as well as 3 farm veterinarians. All participants who completed the study were compensated by payment of a $100 honorarium.

We chose the time period of this study to correspond with the seasonality of swine influenza. In the northern midwestern United States, swine influenza activity is maximal in the late fall and winter (16). Home health nurses visited each participant to administer the study questionnaire, collect an initial preseason blood sample in September 1996, and again to collect a postseason blood sample in April 1997. A total of 114 control serum samples were obtained from a serum bank at the Wisconsin State Laboratory of Hygiene. These samples had been submitted for routine serologic testing from residents of urban Milwaukee, Wisconsin, between August 30, 1996, and March 13, 1997. Because the people from whom control sera were obtained were not specifically enrolled in our study, contacting these persons to gather additional information regarding their health status or activities was neither possible nor ethically appropriate. The use of human participants and control human serum samples in this study was approved by the Human Participant Committees of both the University of Wisconsin-Madison and the Centers for Disease Control and Prevention.

### Questionnaire Topics

Farm participants were questioned as to their age and sex, their overall health, the nature of their contact with swine, and their influenza virus vaccination history. The specific questions asked of each participant are listed in the questionnaire (Figure).



**What is your age?** ____ **Are you:** ☐ Male ☐ Female

**Are you a:**
☐ Farm owner/manager
☐ Farm employee
☐ Family member of a farm owner
☐ Family member of a farm employee

**If you are a manager or employee, how long have you been involved in swine production?**
☐ <1 year
☐ 1-4 years
☐ 5-10 years
☐ >10 years

**How far is your home from the swine farm?**
☐ Live on the swine farm
☐ Live within 1 mile of the farm
☐ Live farther than 1 mile from the farm

**If you live on the farm, how long have you lived on this or any other swine farm?**
☐ <1 year
☐ 1-4 years
☐ 5-10 years
☐ >10 years

**Approximately how often do you go to the swine barn?**
☐ Everyday
☐ 4-6 days/week
☐ 1-3 days/week
☐ Do not go to the barn at all

**On an average day that you go to the barn, approximately how many hours are you in contact with the swine?**
_____

**How often are you in contact with swine other than those on the farm you own or work? (e.g., at a friend's farm, at a fair, at a sale barn, etc.)**
☐ Everyday
☐ Weekly
☐ Several times/year
☐ Rarely
☐ Never

**How often have you received an influenza vaccine (a "flu shot")?**
☐ I've received it regularly (almost every year), starting in 19__.
☐ I've received it occasionally.
☐ I've never received any influenza vaccine.

**Did you receive the swine influenza shot in 1976-1977?**
☐ Yes
☐ No
☐ Unsure

**Have you served in the military since 1975?**
☐ Yes (what years? _____)
☐ No

**Do you suffer from any of the following chronic disease problems?**
☐ Asthma
☐ Chronic bronchitis
☐ Other chronic respiratory disease
☐ Heart disease
☐ Diabetes
☐ Chronic kidney disease
☐ Cancer (please specify type: _____)
☐ Other chronic disease condition not listed above
(please specify type: _____)

Figure. Questionnaire administered to swine farm participants in this study.

### Laboratory Procedures

Each participant was assigned an ID number so that laboratory samples could be assayed without knowledge of personal identifier information. The human serum samples were treated with receptor-destroying enzyme (Denka Seiken Co., Ltd., Tokyo, Japan) at 37°C for 18 h to eliminate nonspecific inhibitors of hemagglutination, after which the samples were tested for HA-specific antibodies using a standard hemagglutination-inhibition (HI) assay (17). The following strains of human and classical swine influenza A viruses were employed as antigens: A/Johannesburg/82/96 (A/JOH; human H1N1), A/Nanchang/933/95 (A/NAN; human H3N2), A/Nebraska/01/92 (A/NEB; zoonotic human isolate of swine H1N1 influenza virus), and A/Swine/Indiana/1726/88 (Sw/IND; swine H1N1). Control sera included sheep anti-human H1 and anti-human H3 subtype-specific sera, ferret anti-A/NEB, and normal sheep serum. Each human serum sample was also assayed without added viral antigen (serum-only control) in parallel with the virus-specific assays. All HI assays were run simultaneously, and HI titers were defined as the reciprocal of the highest dilution of serum that inhibited virus-induced hemagglutination of a 0.5% solution of chicken red blood cells. To calculate geometric mean titers (GMTs) for individual cohorts, we included values for virus-specific titers only if they were greater than the corresponding serum-only control values. Fourfold rises in titer were examined for the study participants by comparing their pre and postseason serum antibody titers.

### Statistical Analyses

The numbers of sera with an HI titer $\geq$40 to either swine virus were compared among the preseason farm participant samples versus the urban control samples by chi-square analysis. The GMTs of the samples from preseason farm participants were compared to the GMTs of the urban control sera by using Wilcoxon rank sum analysis with normal approximation.

We also evaluated associations between preseason seropositivity to swine influenza viruses at HI titers ≥40 or ≥80 among the farm participants and specific aspects of swine exposure or other variables. These associations were then examined for statistical significance by chi-square or two-sided Fisher's exact analyses. Multivariate analysis was not done because of the small numbers of participants with elevated preseason titers to swine influenza viruses (17 participants with titers ≥40; 11 participants with titers ≥80).

## Results

Seventy-nine swine farming participants were initially enrolled, including 20 farm owners (ages 28–59 years), 14 spouses of farm owners (34–57 years), 12 children of farm owners (7–21 years), 21 farm employees (19–43 years), 9 spouses of farm employees (21–39 years), and 3 veterinarians (29–54 years). In total, the farm participants included 44 males of median age 37 (range 13–59 years) and 35 females of median age 34 (range 7–57 years). Of these participants, two people moved out of the area during the study and did not participate further, and three people chose to withdraw from the study before their second blood samples were collected. The preseason samples from these five participants were not included in the analyses. Two participants (both with elevated titers to swine viruses) did not complete the questionnaire. Serologic data from these two participants were included in the comparison of preseason titers of farm participants versus controls but could not be used to assess variables associated with seropositivity to swine viruses. The control sera were from 54 males of median age 32 (range 2–58 years) and 60 females of median age 34 (range 4–54 years).

To interpret the HI titers as showing differences in exposure to swine versus human influenza viruses, we first had to demonstrate that no serologic cross-reactivity in HI assays between the human and swine reference strains existed. We compared HI titers by using virus-specific sheep and ferret reference sera (Table 1) and clearly showed no serologic cross-reactivity in HI assays between the human H1N1, human H3N2, and swine H1N1 viruses. The HI titers to homologous viruses were 320–640, whereas titers to heterologous viruses (H1 vs. H3 or human H1 vs. swine H1) were only 5–10.

The preseason serum samples from 17/74 farm participants had HI titers ≥40 (titer range 40–160) against either A/NEB or Sw/IND; 15/17 were seropositive to both swine viruses. These participants included seven farm owners (range 41–55 years), seven family members of farm owners (range 7–54 years), a 33-year-old farm employee, a 38-year-old family member of a farm employee, and a 43-year-old veterinarian. In contrast, only 1/114 of the urban control serum samples (from a 41-year-old) had a positive HI titer against a swine virus (HI titer=40 against only A/NEB). The difference in the number of seropositive samples between the farm participant and urban control cohorts was statistically significant ($p<0.001$). Similarly, the GMTs of the preseason serum samples from the farm participants to both swine-lineage viruses (A/NEB and Sw/IND) were significantly higher ($p<0.001$) than the GMTs of the samples from the urban control participants (13.2 vs. 5.1 and 15.7 vs. 5.4, respectively). In contrast, the farm participants' GMTs to the reference human H1 (A/JOH) and H3 (A/NAN) viruses were not significantly different from those of the urban control samples (Table 2).

Only three farm participants demonstrated fourfold rises in titer to either of the swine viruses. These rises were not associated with illness in either the human participants or the pigs on their farms.

Each of the variables on the questionnaire (Figure) was investigated for association with preseason sample seropositivity to Sw/IND at HI titers ≥40 or ≥80, A/NEB at HI titers ≥40 or ≥80, and either swine virus at HI titers ≥40 or ≥80. The variables associated with seropositivity to either swine virus at HI titers ≥40 or ≥80 and the statistical strength of those associations are shown in Table 3. (Results for seropositivity to each individual swine virus are not shown but were consistent with the summary statistics presented in Table 3.) Being a farm owner, being part of a farm family (a farm owner or a farm owner's family member), living on a swine farm, and going into a swine barn ≥4 days/week were all associated with seropositivity to swine influenza viruses. Beyond these factors of pig contact, being ≥50 years of age (but not ≥36 years of age, the median age of the farm participants in the study) was associated with swine virus seropositivity; having received the swine flu vaccine in 1976–77 or having ever received any influenza virus vaccine was also associated with swine virus seropositivity. (All four persons who received the swine influenza vaccine also reported having received other influenza virus vaccines.)

## Discussion

Although zoonotic infections with swine influenza viruses have been documented previously (4,15), the results of the present study strongly support the hypothesis that people associated with swine production are infected with swine influenza viruses more regularly than the small number of zoonotic infections in the literature would suggest. Previous studies by Kluska et al. (18), Woods et al. (19), and Schnurrenberger et al. (20) in the 1960s suggested increased rates of infection among persons in contact with pigs or working with swine influenza viruses. In this study, we specifically associated factors related to a person's degree of contact with pigs to seropositivity to swine viruses. The number of hours per day spent in the barn was not a factor of significance, suggesting that the overall frequency of pig contact is a more important consideration than the length of contact at any one time. This lack of significance is consistent with the fact that influenza virus infections in pigs occur sporadically, and pigs generally only shed virus for approximately 7 days after infection (21). During the course of this study, pigs on only one farm were reported to exhibit signs of influenza-like illness. Influenza viruses were not isolated from nasal swab samples collected from these pigs.

Table 1. Hemagglutination-inhibition titers of control sera to reference virus strains used in this study

| Control serum | Reference influenza A viruses | | | |
|---|---|---|---|---|
| | A/Johannesburg/82/96 (A/JOH) (human H1N1 virus) | A/Nanchang/933/95 (A/NAN) (human H3N2 virus) | A/Nebraska/01/92 (A/NEB) (zoonotic swine H1N1 virus) | A/Swine/Indiana/1726/88 (Sw/IND) (swine H1N1 virus) |
| Sheep anti-human H1N1[a] | 640 | 10 | 5 | 5 |
| Sheep anti-human H3N2[b] | 5 | 320 | 5 | 5 |
| Ferret anti-A/NEB | 5 | 10 | 640 | 320 |
| Ferret anti-swine H1N1[c] | 5 | 10 | 320 | 640 |
| Normal sheep serum | 10 | 10 | 5 | 5 |

[a]Produced by immunization of sheep with A/Taiwan/1/86 and A/Texas/36/91 (H1N1).
[b]Produced by immunization of sheep with A/Shangdong/9/93, A/Johannesburg/33/94, and A/Nanchang/933/95 (H3N2).
[c]Produced by immunization of ferrets against A/Swine/Wisconsin/01/88 (H1N1).

Two factors not directly related to swine contact were also statistically associated with seropositivity to swine viruses in our study. First, being ≥50 years of age was associated with swine virus seropositivity. In an earlier study, Schnurrenberger et al. (20) collected samples in 1966 from abattoir workers, pork producers, swine exhibitors at a state fair, and veterinarians; they also found an association between age and seropositivity to a classical H1N1 swine influenza virus. In that study, the major impact of age was apparent for people born before 1920, suggesting an effect from exposure to the swine-like 1918 pandemic influenza virus (22–24). We could not fully separate the effects of age and exposure over time to swine. All of the participants ≥50 years of age were farm owners or farm family members. However, several factors indicate that, although age may play a role in seropositivity to swine viruses, exposure to swine is a more dominant factor. Farmers and their family members were significantly more likely than employees and their family members to have elevated titers to swine viruses; farmers and their families were also more likely to have exposure to swine and to be exposed over a longer period of time. Specifically, 88% of the farm owners and their families lived on the swine farm, compared to 7% of the employees and their families. Furthermore, of the farm owners and their families who lived on the farm, 77% had lived there >11 years. Farm owners had significantly more years in swine production than their employees (Mantel-Haenszel chi-square test for trend, $p<0.001$). Among younger study participants (<50 years of age), 21% and 18% of the farm family members had titers of ≥40 and ≥80 to swine viruses, respectively, compared to only 7% and 4% for employees and their family members. Although these differences among the younger participants were not statistically significant ($p=0.09$ to 0.14), they are consistent with the pattern of elevated titers seen among those with a higher level of exposure to pigs. Finally, our control population was of the same overall age distribution as our farm participants, yet only a single 41-year-old person among these controls was seropositive to a swine virus.

A second factor unrelated to swine contact significantly associated with swine virus seropositivity was having received either the swine influenza virus human vaccine in 1976–77 or ever having received any human influenza virus vaccine. However, vaccination status alone most likely did not determine seropositivity to swine viruses among our farm participants. Vaccination was only associated with seropositivity at a titer ≥40, but not at ≥80. Although we do not have historical data for our urban control samples, we have no a priori reason to suspect that these people would have had substantially different vaccination histories. However, only 1/114 of these participants demonstrated a titer ≥40 to a swine virus. Likewise, the proportions of employees and their family members who received the swine influenza vaccine (4%) or other influenza vaccines (32%) were not significantly different from the proportions of farm family members who received the swine influenza vaccine (6%) or other influenza vaccine (22%), but farm family members were significantly more likely to have elevated titers to swine viruses. Regarding having received the 1976–77 swine influenza vaccine, antibodies produced against that vaccine would not likely be present at detectable levels 20 years later. However, studies have shown that vaccination with more recent human influenza A (H1N1) viruses can boost

Table 2. Geometric mean titers of preseason serum samples from farm participants and urban control serum samples

| Participants | Reference influenza A viruses | | | |
|---|---|---|---|---|
| | A/Johannesburg/82/96 (A/JOH) (human H1N1 virus) | A/Nanchang/933/95 (A/NAN) (human H3N2 virus) | A/Nebraska/01/92 (A/NEB) (zoonotic swine H1N1 virus) | A/Swine/Indiana/1726/88 (Sw/IND) (swine H1N1 virus) |
| Farm participants | 15.3 | 8.6 | 13.2[a] | 15.7[a] |
| Urban control participants | 14.2 | 8.0 | 5.1 | 5.4 |

[a]$p>0.0001$ (Wilcoxon rank sum analysis with normal approximation).

Table 3. Variables associated with seropositivity to swine influenza viruses among the farm participants and the statistical strength of these associations[a]

| Variable | HI titer ≥40[b] | HI titer ≥80[b] |
|---|---|---|
| Being a farm owner | p=0.04 | p=0.02 |
| Being a farm owner or the family member of a farm owner | p=0.03 | p=0.02 |
| Living on a swine farm | (p = 0.07) | p=0.04 |
| Going into a swine barn ≥4 days/wk | (p = 0.12) | p=0.04 |
| Age ≥50 yrs | p=0.02 | p=0.03 |
| Having received the swine flu vaccine in 1976–77 | p=0.02 | (p = 0.44) |
| Ever having received any influenza virus vaccine | p=0.03 | (p = 0.19) |

[a]p values determined by chi-square or two-sided Fisher's exact analyses; p values >0.05 cut-off for significance are shown in parentheses.
[b]To either swine virus. Abbreviation used: HI, hemagglutination-inhibition.

titers to swine-like viruses in those previously exposed (25). Therefore, the statistical association between seropositivity to swine viruses and vaccination likely reflects a vaccine-induced boosting of antibody titers in persons previously exposed to a swine influenza virus. Because of the overall low numbers of participants with elevated titers to swine viruses, we were not able to perform meaningful multivariate analysis of the data to definitively segregate the effect of vaccination history (or age) from other variables.

Because a relatively small number of zoonotic swine influenza virus infections have been documented by virus isolation, whether infections with swine influenza viruses are clinically different than infections with routine human influenza viruses remains unclear. Our data suggest that aggressively pursuing virus isolation when people involved in swine farming have influenza-like illnesses would be valuable. In this way, retrospective studies of the clinical appearance of a larger number of zoonotic swine influenza cases may be possible.

Our findings suggest a second issue. Pigs may serve as hosts for the adaptation of avian viruses to replication in mammalian species (26). In addition, pigs are clearly recognized as hosts in which genetic reassortment between human and avian viruses can produce novel strains of pandemic potential (7,11,12). While this concern has historically been thought to be most important in the "Asia epicenter" (12,27), avian H1N1 viruses have spread widely within the swine population of northern Europe since 1979 (7,28–31), avian H4N6 viruses were isolated from pigs in Canada in 1999 (32), and human/swine/avian reassortant H3N2 (33–35) and H1N2 (36,37) viruses have spread widely within the swine population of the United States since 1998. Given the apparent frequency with which swine farm workers in our study were exposed to influenza viruses from pigs, more closely monitoring such persons as potential sentinels for the emergence of novel influenza viruses from the swine populations of developed countries with extensive swine-raising industries may be prudent.

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# Phylogenetic Relationships of Southern African West Nile Virus Isolates

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Phylogenetic relationships were examined for 29 southern African West Nile virus (formal name *West Nile virus* [WNV]) isolates from various sources in four countries from 1958 to 2001. In addition sequence data were retrieved from GenBank for another 23 WNV isolates and Kunjin and Japanese encephalitis viruses. All isolates belonged to two lineages. Lineage 1 isolates were from central and North Africa, Europe, Israel, and North America; lineage 2 isolates were from central and southern Africa and Madagascar. No strict correlation existed between grouping and source of virus isolate, pathogenicity, geographic distribution, or year of isolation. Some southern African isolates have been associated with encephalitis in a human, a horse, and a dog and with fatal hepatitis in a human and death of an ostrich chick.

West Nile virus (formal name *West Nile virus* [WNV]) is a mosquito-borne member of the *Flaviviridae* family (genus *Flavivirus*), which was originally isolated from the blood of a febrile patient in Uganda in 1937 (1). The virus is widely distributed in Africa, Asia, and Europe and was recently spread to the Western Hemisphere, where its presence was recognized in the northeastern United States in 1999 (2,3).

After the initial isolation of the virus, sporadic cases and outbreaks of febrile disease were recorded in humans in Africa, the Near East, and Asia; the largest outbreaks occurred in Israel in 1950–1954 and 1957 and in South Africa in 1974 (4,5). Meningoencephalitis was first observed in elderly patients in Israel in 1957 and subsequently observed as a complication in young children in India (6,7). In 1962, the isolation of WNV from a horse with encephalitis was reported in Egypt; from 1962 through 1966, meningoencephalitis occurred in both humans and horses in a series of outbreaks in southern France (8,9). In 1983, four cases of hepatitis, two fatal, were attributed to WNV infection in the Central African Republic (CAR), a report that has been largely overlooked (10). A marked increase in the frequency and severity of outbreaks of human disease during the 1990s followed, often involving horses; epidemics occurred in Algeria, Romania, Morocco, Tunisia, Italy, Russia, France, Israel, and the United States (11–18). Moreover, the recent outbreaks in Romania, Israel, and the United States were characterized by concurrent deaths in birds (19,20). The virus circulating in the United States was found to be most closely related genetically to a WNV isolate associated with goose deaths in Israel in 1998, suggesting that the virus was imported into America from the Near East, either in an infected bird, mosquito, human, or other animal. The exact mechanism of the introduction will probably remain unknown (18,19).

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In southern Africa, WNV was found to be widely endemic in areas where the principal vector, *Culex univittatus*, and avian hosts of the virus are present. Human infections tended to be sporadic; large epidemics occurred when unusually high rainfall or hot weather favored breeding of the vectors (21–23). Outbreaks were associated with concurrent epizootics in birds, as evidenced by antibody studies, and 13 species of experimentally infected wild birds supported replication of the virus without becoming overtly sick or dying (24–26). The largest epidemic occurred in 1974 and involved tens of thousands of human cases over a 2,500-km$^2$ area of the Karoo and Northern Cape Provinces (5,21). A mean antibody prevalence of 55% in humans was recorded in the affected region after the outbreak; levels of 80% to 85% were recorded in some locations. In one town, 1,700 people sought medical attention. Infections were most frequently subclinical or associated with mild febrile illness characterized by rash, myalgia, and arthralgia. No human deaths were recorded, and no excess bird deaths were observed, although an antibody prevalence of 53% was detected in wild birds after the outbreak (5,21). A smaller epidemic occurred from 1983 through 1984 in association with an outbreak of Sindbis virus (formal name: *Sindbis virus*) infection in the Witwatersrand-Pretoria region of South Africa, and again no deaths were recorded (23). Since then, the number of human WNV infections confirmed in South Africa, mainly on the basis of detection of antibody response, has remained fairly constant at approximately 5–15 cases per year. Only a proportion of suspected cases are subject to laboratory investigation. Despite the apparently low level of virus activity, however in recent years, a few isolations of WNV have been made from patients with severe disease, including a fatal case of hepatitis in 1989 and nonfatal encephalitis in 2001 (National Institute for Communicable Diseases [NICD], unpub. data).

The apparent increases in the frequency of neurologic infections, human case-fatality rates, and horse and bird deaths

in the Northern Hemisphere raised the question of whether a recent emergence of WNV strains with increased pathogenicities occurred or whether the virulence of the virus had previously been underestimated. Investigations with hemagglutination-inhibition kinetics, titer ratios from cross-neutralization tests, reactivity to monoclonal antibodies, and cDNA/RNA heteroduplex restriction enzyme digest profiles confirmed that strain differences occur but did not establish any links between variants and pathogenicity (27–30). More recently, phylogenetic analyses based on different regions of the genome have shown that WNV isolates form two well-supported lineages (19,31,32). Lineage 1 includes viruses from Africa north of the equator, Europe, Asia, and North America; Kunjin virus (formal name: Kunjin virus [KUNV]) from Australia constitutes a subtype of this lineage. Lineage 2 consists solely of viruses from Africa and Madagascar. These findings support the emergence of increased virulence in lineage 1. Lineage 2 isolates are thought to be associated with endemic infection of low virulence in Africa (18,19).

The South African prototype WNV isolate, H 442, was obtained in 1958 from the blood of a person with mild febrile disease who had been bitten by mosquitoes while catching birds in mist nets for arbovirus studies (33). This isolate is the only one from South Africa to have been included in a phylogenetic study, and its characterization as a member of lineage 1 (19) seems to be inconsistent with findings for other African isolates from south of the equator. However, apart from recent isolation of WNV from severe cases of human disease in southern Africa, isolations of the virus were associated with a fatal infection in a dog, a horse, and an ostrich chick (34; unpub. data, NICD). Hence, we were prompted to undertake phylogenetic investigation of southern African WNV isolates.

## Methods and Materials

### Virus Isolates

Phylogenetic analysis was performed on partial nucleotide sequence data from 52 WNV isolates. This included 29 isolates obtained from various sources in southern Africa from 1958-2001, which were sequenced for the study (Table 1). Twenty-three WNV isolates and KUNV and Japanese encephalitis viruses (JEV) for which sequence data were retrieved from GenBank were also analyzed (Table 2). Southern African isolations made at NICD included 8 human, 15 mosquito, 1 bird, and 2 sentinel animal isolates. Three isolates obtained by the Onderstepoort Veterinary Institute, Pretoria, included the horse and ostrich isolates, which originated from the Regional Veterinary Laboratory, Stellenbosch, Western Cape Province, and a dog isolate, which originated from the Veterinary Investigation and Research Laboratory, Gaborone, Botswana, and was initially described as *Wesselsbron virus* (WESSV) but was later found to be WNV (34; BJH Barnard, pers. comm.). No isolates from the 1974 epidemic could be located for this study. The isolates were stored at -70°C as freeze-dried 10% mouse brain suspensions, and low-passage material was selected for sequencing (Table 1). With the prototype isolate, H 442, stocks of freeze-dried material were sequenced at various mouse passage levels (2–7), and passaged 2 material was passaged 10 times in mice and sequenced.

The bird, mosquito, and sentinel animal isolates (Table 1) were obtained during epidemiologic studies (21); the human isolates (Tables 1 and 3) were obtained from clinical specimens submitted to the Arbovirus Unit or the Special Pathogens Unit at NICD for the investigation of suspected cases of arbovirus infection or for the exclusion of African viral hemorrhagic fevers. In all instances, WNV was isolated from human serum samples by mouse inoculation, except for patient 5, from whom the virus was isolated from a liver sample taken at autopsy.

### Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Nucleotide Sequencing of Amplicons

Freeze-dried mouse brain suspensions were reconstituted in water, and viral RNA was extracted for the RT-PCR by using the QIAamp Viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A 255-bp region of the E glycoprotein gene (genome positions 1402–1656) was amplified with primers designated WN132 and WN240, as described by Berthet et al. (32). The RT-PCR reactions were performed with the TITAN One Tube RT-PCR kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. The nucleotide sequences of the amplicons were determined with BigDye Terminator Cycle Sequencing Ready Reaction kits with AmpliTaq DNA polymerase FS (Applied Biosystems, Warrington, Great Britain) according to the manufacturer's instructions. Sequences were obtained for both strands of the DNA amplicons by using each primer, WN132 and WN240, for confirmation of the nucleotide sequence. Products were purified by using Centri-Sep spin columns (Princeton Separations Inc., Adelphia, New Jersey) and analyzed with a 377 GenAmp automatic sequencer (Applied Biosystems).

### Phylogenetic Analysis

Editing and alignment of the nucleotide sequence data were performed with DNASIS for Windows Version 2.5 (Hitachi Software Engineering America, Brisbane, CA). The phylogenetic analysis was performed on a 227-bp region of the amplicons with a neighbor-joining distance method (unordered "p" parameter model), with Phylogenetic Analysis with Parsimony (PAUP) software version 4.0b4a for Macintosh (35). Bootstrap confidence intervals were calculated by 500 heuristic search replicates.

## Results

### Clinical Features of WNV Infections

The human isolates (Tables 1 and 3) were obtained from clinical specimens submitted to the Arbovirus Unit for the investigation of suspected cases of arbovirus infection or undi-

Table 1. Twenty-nine southern African West Nile virus isolates

| Isolate | Yr of isolation | Passage level | Source | Location | GenBank Accession no. |
|---|---|---|---|---|---|
| H 442 | 1958 | m2 | human | Ndumo, Kwa-Zulu Natal, RSA[a] | AF514918 |
| H 912 | 1964 | m4 | human | Middelburg, Mpumulanga, RSA | AF514919 |
| H 1127 | 1968 | m4 | human | Johannesburg, Gauteng, RSA | AF514920 |
| SPU 101/89 | 1989 | m5 | human | Bloemfontein, Free State, RSA | AF514921 |
| SPU 116/89 | 1989 | m3 | human | Pretoria, Gauteng, RSA | AF514922 |
| SPU 167/89 | 1989 | m7 | human | Ovambo, Namibia | AF514923 |
| SA 381/00 | 2000 | m1 | human | Naboomspruit, Northern Province, RSA | AF514944 |
| SA 93/01 | 2001 | m1 | human | Johannesburg, Gauteng, RSA | AF514945 |
| An 2842 | 1958 | m3 | long-billed crombec | Ndumo, Kwa-Zulu Natal, RSA | AF51424 |
| An 15228 | 1968 | m4 | sentinel pigeon | Olifantsvlei, Gauteng, RSA | AF514925 |
| An 20587 | 1972 | m3 | sentinel hamster | Mopeia, Mozambique | AF514926 |
| An 24630 | 1977 | m5 | dog | Gabarone, Botswana | AF514927 |
| 94034039 | 1994 | m4 | ostrich | Prince Albert, Western Cape, RSA | AF514928 |
| 9604058 | 1996 | m4 | horse | Somerset West, Western Cape, RSA | AF514946 |
| Ar 4064 | 1962 | m2 | *Culex theileri* | Olifantsvlei, Gauteng, RSA | AF514929 |
| Ar 4821 | 1962 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514942 |
| Ar 5254 | 1963 | m4 | *Cx. univittatus* | Welkom, Free State, RSA | AF514930 |
| Ar 5995 | 1963 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514943 |
| Ar 6127 | 1964 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514931 |
| Ar 6129 | 1964 | m4 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514932 |
| Ar 6618 | 1964 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514933 |
| Ar 7941 | 1965 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514934 |
| Ar 7943 | 1965 | m2 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514935 |
| Ar 7944 | 1965 | m3 | *Cx. theileri* | Olifantsvlei, Gauteng, RSA | AF514936 |
| Ar 8352 | 1966 | m3 | *Cx. univittatus* | Olifantsvlei, Gauteng, RSA | AF514937 |
| Ar 10825 | 1969 | m2 | *Cx. univittatus* | Bethulie, Free State, RSA | AF514941 |
| Ar 10864 | 1969 | m2 | *Cx. univittatus* | Bethulie, Free State, RSA | AF514938 |
| Ar 10893 | 1969 | m4 | *Aedes caballus* | Bethulie, Free State, RSA | AF514939 |
| Ar 20758 | 1984 | m4 | *Cx. univittatus* | Rondebult, Gauteng, RSA | AF514940 |

[a]RSA, Republic of South Africa

agnosed fever, except for the three isolates obtained from specimens submitted in 1989 from patients 4, 5, and 6 (Table 3) for the exclusion of African viral hemorrhagic fevers; tests for Marburg disease, Ebola fever, Crimean-Congo hemorrhagic fever, Rift Valley fever, Lassa fever, and hantaviruses were negative.

Patients 1–3 and 6–7 (Table 3) had benign WNV infections with fever, rash, myalgia, and arthralgia; specimens from patient 6 were submitted for the exclusion of viral hemorrhagic fever only because he had an outdoor occupation in Namibia with potential exposure to ticks, and thus Crimean-Congo fever was considered a possibility. Patient 4 also had an outdoor occupation, in Free State Province of South Africa and a definite history of exposure to mosquito bites. During the second week of a febrile illness, he had coagulopathy with abnormal prothrombin index and partial thromboplastin time, hemoglobinuria, pancreatitis, and renal failure requiring dialysis. He made a prolonged but full recovery.

Patient 5, who lived on the northern outskirts of Pretoria, had fever, nausea and vomiting, epigastric pain, elevated blood and urine amylase, elevated blood urea and creatinine values, and markedly elevated transaminases. He was admitted to the hospital on the second day of illness with low fever and tender epigastrium. Tests for hepatitis A, B, and C and HIV were negative, and the patient died on the fourth day of illness. No lesions of the pancreas were observed at autopsy, but a massive liver necrosis was found, and WNV was isolated from a liver sample.

Table 2. Twenty-three West Nile virus isolates, plus Kunjin and Japanese encephalitis viruses[a]

| Isolate | Yr of isolation | Source | Location | GenBank accession no. |
|---|---|---|---|---|
| HEg 101 | 1951 | Human | Egypt | AF001568 |
| TL 443 | 1952 | na | Israel | AF205881 |
| G 16919 | 1955 | na | India | AF205885 |
| MP 22 | 1959 | *Coquilletidia metallica* | Uganda | AF001562 |
| PaH 651 | 1965 | Human | France | AF001560 |
| ArB 310 | 1967 | *Culex* sp | CAR | AF001566 |
| ALG-ArDjanet | 1968 | *Culex* sp | Algeria | AF001567 |
| AnMg 798 | 1978 | *Coracopsis vasa* | Madagascar | AF001559 |
| AnD 27875 | 1979 | *Galago senegalensis* | Senegal | AF001569 |
| ArA 3212 | 1981 | *Cx. guiarti* | Ivory Coast | AF001561 |
| HB 83P55 | 1983 | Human | CAR | AF001557 |
| ArMg 956 | 1986 | *Cx. quinquefasciatus* | Madagascar | AF001564 |
| ArMg 978 | 1988 | *Cx. univittatus* | Madagascar | AF001574 |
| HB 6343 | 1989 | Human | CAR | AF001558 |
| ArD 78016 | 1990 | *Aedes vexans* | Senegal | AF001556 |
| ArD 93548 | 1993 | *Cx. neavei* | Senegal | AF001570 |
| 097-50 | 1996 | *Cx. pipiens* | Romania | AF205880 |
| 96-111 | 1996 | na | Morocco | AF205884 |
| Isr 98-Goo1 | 1998 | goose | Israel | AF205882 |
| PaAn981 | 1998 | na | Italy | AF205883 |
| KN 3829 | 1998 | *Cx. univittatus* | Kenya | AF146082 |
| NY-99, 382-99 | 1999 | Chilean flamingo | USA | AF196835 |
| ArNa1047 | unknown | *Ae. albothorax* | Kenya | AF001571 |
| Kunjin | na | na | Australia | AF001572 |
| JE SA 14 | 1954 | mosquitoes | China | U04522 |

[a]Nucleotide sequence data were obtained from GenBank for inclusion in the present phylogenetic analysis; na, not available; CAR, Central African Republic.

Patient 8 lived near NICD, a WNV-endemic focus with artificial lakes and dams, reed beds, mosquitoes, and large bird colonies. The patient was admitted to the hospital with a 2-day history of headache, fever, nausea, and dizziness. She had marked light sensitivity and terminal meningism. Cerebrospinal fluid cultures and blood cultures were negative. She had severe arthralgia, and after discharge from the hospital, a rash developed. She made an uneventful recovery. Laboratory investigation of her illness, with consequent recognition of WNV infection, was probably influenced by the fact that she was a relative of a member of staff of NICD; other cases of WNV-induced encephalitis may have been missed.

The dog from which a WNV isolate was obtained in Botswana (Table 1) was missing for 4 days and found in extremis. The dog had severe diarrhea, became comatose, had convulsions, and died (34). The dog was initially thought to have rabies, and subsequently a flavivirus isolated from brain tissue was thought to be WESSV, but ultimately was shown to be WNV (34; BJH Barnard, pers. comm.). The horse isolate (Table 1) was obtained from the brain of a 6-month-old Thoroughbred foal from a farm in Somerset West District, which died after exhibiting signs of nervous disease. The ostrich chick came from the Prince Albert district in the Western Cape Province and was part of a major ostrich farming area where death in young birds at approximately 2 to 3 weeks of age has been a problem in recent years.

### Genetic Relationships

A phylogenetic tree was generated from sequence data of 52 WNV isolates from 19 countries, plus KUNV and JEV, by neighbor-joining distance analysis with node values generated by 500 bootstrap replications (Figure). The topology shows two distinct lineages. Lineage 1 includes 16 WNV isolates from 13 countries in Europe, Africa, the Near East, India, and the United States, and KUNV from Australia. The Indian isolate and Kunjin virus appear in lineage 1 as monophyletic sister clades. Excluding the Kunjin virus, the maximum nucleotide sequence divergence exhibited in lineage 1

Table 3. Southern African human patients from whom West Nile virus isolates were studied

| Patient | Isolate designation | Yr | Sex/Age | Syndrome | Outcome |
|---|---|---|---|---|---|
| 1 | H 442 | 1958 | M/26 | Fever, rash, myalgia, arthralgia | Survived |
| 2 | H 912 | 1964 | M/Ad[a] | Fever, rash, myalgia, arthralgia | Survived |
| 3 | H 1127 | 1968 | F/Ad | Fever, rash, myalgia, arthralgia | Survived |
| 4 | SPU 101/89 | 1989 | M/33 | Fever, coagulopathy, hemoglobinuria, renal failure | Survived |
| 5 | SPU 116/89 | 1989 | M/27 | Necrotic hepatitis | Died |
| 6 | SPU 167/89 | 1989 | M/22 | Fever, rash, myalgia, arthralgia | Survived |
| 7 | AR 381/00 | 2000 | F/68 | Fever, rash, myalgia, arthralgia | Survived |
| 8 | AR 93/01 | 2001 | F/21 | Fever, rash, myalgia, encephalitis | Survived |

[a]Ad, adult.

(21.5%), was between the Indian isolate G 167919 and CAR isolate ArB 310. Otherwise, the divergences in lineage 1 ranged from a maximum of 10.6% between CAR isolate ArB 310 and Ivory Coast isolate ArA 3212, to a maximum homology of 99.6% between a Senegal isolate AnD 27875 and Algerian isolate Ar/Djanet.

Lineage 2 includes 36 isolates from 7 countries in Africa, plus Madagascar. The maximum divergence of nucleotide sequences in lineage 2 (18.9%) was between a bird isolate from Madagascar AnMg 798 and several South African isolates from humans and mosquitoes. In the South African isolates, homology ranged from 86.3% to 100%. In some instances, identical isolates originated from different sources, years of isolation, or regions. The geographic dispersal of similar isolates was illustrated by the approximately 99.6% homology of Senagalese isolate ArD 78016 and several South African isolates. In contrast, geographic overlap of divergent isolates was also evident, as illustrated by the presence of strains from CAR, Senegal, and Kenya in both lineages.

## Discussion

The genetic relationships determined for isolates included in this study were consistent with previous publications in which isolates from different parts of the world fell into two lineages; one included a few African isolates and all the European, Asian, and North American isolates; the second lineage includes African and Madagascan isolates exclusively. In contrast to a previous finding (19), however, we determined that the South African prototype isolate, H 442, belonged to lineage 2 along with the other southern African isolates. Our findings were consistent in tests with material of different mouse passage levels, including material that had been stored at mouse passage level 2 at the time that the original isolation was made, supporting the conclusion that isolate H442 may have been misidentified in a laboratory abroad. Although no isolates from the 1974 South Africa epidemic could be located for inclusion in this study, they probably belong to lineage 2. All 29 southern African isolates tested belonged to lineage 2, even those that were isolated up to 16 years before or up to 19 years after the epidemic.

The results of this phylogenetic study support previous conclusions that the close relationships between certain isolates from different countries and continents are compatible with local and long-range dispersal of the virus by migratory birds. On the other hand, the divergence between isolates of the two lineages indicates that different strains are circulating

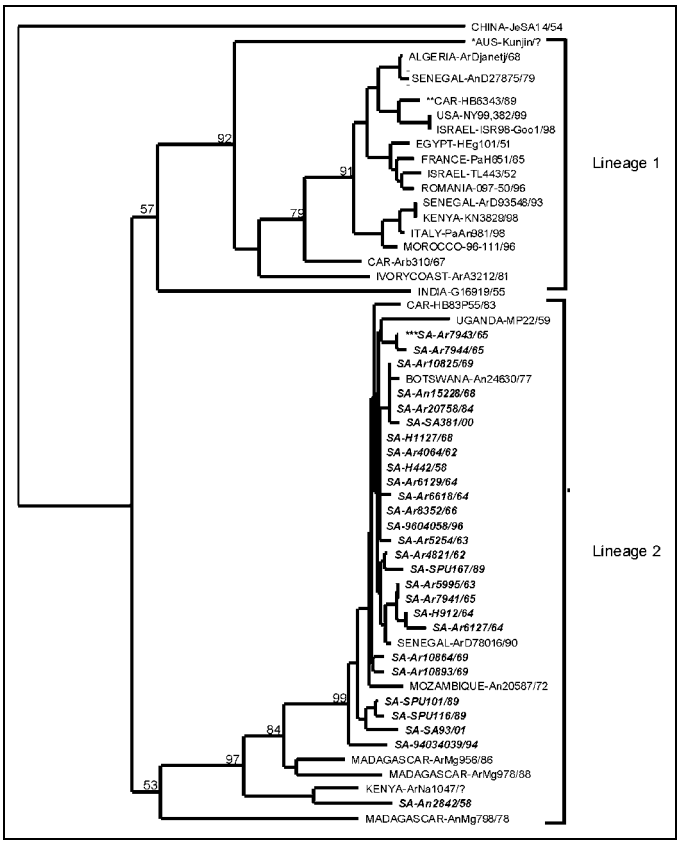


Figure. Genetic relatedness of geographically distinct West Nile isolates determined by using the nucleotide sequence data from a 227-bp region of the E gene. The tree was constructed with PAUP (35) by using neighbor-joining distance program. Node values were determined from 500 replicates. Isolates are labeled: country of isolation-strain identification/year of isolation. GenBank accession numbers are provided in Table 1 and 2. (*AUS, Australia; **CAR, Central African Republic; ***SA, South Africa). Sequences derived from West Nile virus isolates determined in the Special Pathogens Unit are shown in bold, italic type.

in some countries, such as CAR, Senegal, and Kenya. No genetic distinction appears between strains circulating in enzootic cycles and human outbreaks, and the wide range of suitable vector hosts probably facilitates the dispersal of WNV. The two extremes of bird migration routes, southern Africa and Eurasia, have divergent strains, whereas the central regions have a mixture of lineages 1 and 2. One possible explanation is that, because of the distances involved, the birds probably could not remain viremic from one extreme of their migratory route to the other. Ultimately viruses will likely pass from one region to the other.

Benign West Nile fever in humans is a febrile illness, with myalgia, arthralgia, lymphadenopathy, and often maculopapular or roseolar rash. Other documented signs and symptoms include nausea, vomiting, diarrhea, conjunctivitis, abdominal pain, pancreatitis, myocarditis, and hepatosplenomegaly. Few patients have serious complications such as acute aseptic meningitis, encephalitis, or necrotic hepatitis. Although many patients are reported to have severe illness with high case-fatality rates (4% to 13.3%) in recent epidemics in the Northern Hemisphere (16), serious disease occurs in 1% of infections (12,14,36,37). Moreover, most fatalities have been recorded in elderly or immunocompromised patients. The recent outbreaks have raised questions regarding strain variation and the possible emergence of enhanced pathogenicity. However, during a 1957 WNV outbreak in Israel, the death rate was 8.2% (4/ 49) in a group of elderly patients and 8.4% (35/417) in a epidemic in the same country in 2000 (16). Thus, the perceived virulence of the virus in recent epidemics may partly be because of the emergence or reemergence of existing strains of WNV in geographic locations with immunologically naive populations, high medical alertness, and active surveillance programs. When antigenic and molecular studies failed to demonstrate differences between WNV isolates from patients with hepatitis and benign disease in the CAR, rather than ascribing differences in clinical manifestation to virus strain variation, a new definition of disease spectrum to include liver involvement was considered (10,30).

The fact that no cases of severe disease were recognized in the large numbers of patients seen during the 1974 WNV epidemic in South Africa could indicate that isolates of lineage 2 lack virulence, but little clinical awareness of the pathogenic potential of arboviruses in general may also have played a role. The WNV epidemic coincided with the start of a major Rift Valley fever (RVF) epidemic in South Africa. Only in the next year (1975), when publicity surrounding the occurrence of Marburg disease in the country alerted clinicians to hemorrhagic disease, were specimens from hospitalized patients with unrecognized infections submitted for laboratory investigation (38). The invesigation proved for the first time that RVF could be a fatal disease in humans. Moreover, the isolations of WNV from patients with severe disease in recent years, including a fatal case of hepatitis and nonfatal mild meningoencephalitis during a quiescent period in virus activity, confirm that strains of lineage 2 can be pathogenic. Diagnosis of these cases owed more to the availability of appropriate laboratory services than to clinical recognition of WNV infection. Clearly, a need for greater awareness of the variety of symptoms, including hepatitis, associated with WNV throughout its distribution range is needed.

The occurrence of encephalitis in a dog in Botswana was followed by a serosurvey and pathogenicity trials in South Africa; the conclusion was that dogs are subject to WNV infection but probably do not play an important role in the epidemiology of the disease (39). The occurrence of WNV in horses in southern Africa is the subject of an ongoing investigation, but the recognition of an equine case of encephalitis remains an isolated event.

Although bird deaths characterized recent epidemics in the Northern Hemisphere, the disease appears to have spared African species in a New York zoo (19,20,40). Hooded crows had previously been shown to be susceptible to experimental infection with an Egyptian isolate (41), but experimentally infected adult wild birds of 13 species in South Africa had viremic infection without overt disease (24–26). The only death observed from an experimental infection in South Africa occurred in day-old domestic chicks; susceptibility declined with increasing age (PG Jupp, pers. comm.). WNV infection may have been responsible for the deaths observed in young ostrich chicks in the Western Cape Province, where the virus was isolated from a dead chick (Table 1), but many viruses, bacteria, intestinal parasites, and nutritional factors contribute to the death of young birds, and investigations are continuing. In conclusion, increased veterinary awareness of the pathogenic potential of the virus is needed.

Dr. Burt is a specialist in the Special Pathogens Unit at the National Institute for Communicable Diseases, South Africa. She is involved in the diagnosis and investigation of viral hemorrhagic fevers and arboviruses.

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# Pandrug-Resistant *Acinetobacter baumannii* Causing Nosocomial Infections in a University Hospital, Taiwan

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The rapid emergence (from 0% before 1998 to 6.5% in 2000) of pandrug-resistant *Acinetobacter baumannii* (PDRAB) was noted in a university hospital in Taiwan. To understand the epidemiology of these isolates, we studied 203 PDRAB isolates, taken from January 1999 to April 2000: 199 from 73 hospitalized patients treated at different clinical settings in the hospital and 4 from environmental sites in an intensive-care unit. Pulsed-field gel electrophoresis analysis and random amplified polymorphic DNA (RAPD) generated by arbitrarily primed polymerase chain reaction of these 203 isolates showed 10 closely related genotypes (10 clones). One (clone 5), belonging to pulsotype E and RAPD pattern 5, predominated (64 isolates, mostly from patients in intensive care). Increasing use of carbapenems and ciprofloxacin (selective pressure) as well as clonal dissemination might have contributed to the wide spread of PDRAB in this hospital.

The emergence and rapid spread of multidrug-resistant isolates causing nosocomial infections are of great concern worldwide (1–5). Although methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and extended-spectrum β-lactamase and AmpC–producing *Enterobacteriaceae* have been the subject of much of this attention, multidrug resistance among some non-*Enterobacteriaceae* organisms, such as *Acinetobacter baumannii*, has also emerged (1–10).

During the last decade, nosocomial infections caused by multidrug-resistant *A. baumannii* have been reported (3,4,6,7,11–13). Initial concern about carbapenem-resistant *A. baumannii* (CRAB) began when the first nosocomial outbreak occurred in the United States in 1991 (6). Since then, CRAB infections and hospitalwide outbreaks have been reported from many other countries (7,11,14–17).

In May 1998, the first isolate of CRAB—which was also resistant to almost all commercially available antibiotics, including all cephalosporins, aztreonam, aminoglycosides, and ciprofloxacin (pandrug-resistant *A. baumannii*, PDRAB)—was recovered from a leukemia patient with bacteremia in an oncology ward. Three more isolates of PDRAB were recovered from three patients admitted to three general wards in January–February 1999. Since April 1999, clusters of PDRAB isolates were found in patients infected or colonized by these organisms throughout the hospital, particularly in patients hospitalized in several intensive-care units (ICUs). The outbreak persisted for more than 12 months, beginning April 1999, and involved 73 patients. The aim of our study was to document the emergence of PDRAB in a university hospital and to characterize a hospitalwide outbreak due to PDRAB by investigating antibiotypes and genotypes by pulsed-field gel electrophoresis (PFGE) and arbitrarily primed polymerase chain reaction (APPCR).

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## Materials and Methods

### Background

National Taiwan University Hospital (NTUH) is a 2,000-bed hospital located in northern Taiwan. The Nosocomial Infection Control Committee of the hospital was established in 1980. Since then, identification of pathogens that cause nosocomial infections and collection and analysis of antimicrobial susceptibility results of these pathogens from the hospital's clinical microbiology laboratory have been performed (4). Definitions for nosocomial infection followed the guidelines of the National Nosocomial Infections Surveillance system (4,18).

To determine the secular trend of CRAB, we analyzed data on the disk-diffusion susceptibilities to imipenem of this organism recovered in the period 1993–2000 in NTUH. Organisms were categorized as susceptible, intermediate, or resistant to the antimicrobial agents tested on the basis of guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS) (19). PDRAB described isolates resistant to almost all commercially available antibiotics tested (i.e., ceftazidime, cefepime, ticarcillin-clavulanate, piperacillin-tazobactam, aztreonam, imipenem, meropenem, gentamicin, amikacin, ofloxacin, and ciprofloxacin). Isolates of CRAB, which did not belong to PDRAB, were usually susceptible to ciprofloxacin, ofloxacin, gentamicin, or amikacin.

The annual use of carbapenems (imipenem and meropenem), extended-spectrum cephalosporins (cefotaxime,

ceftriaxone, ceftazidime, cefepime), aminoglycosides (gentamicin, tobramycin, netilmicin, and amikacin), and ciprofloxacin, expressed as grams per 1,000 patient-days from 1993 to 2000, was also analyzed. Imipenem was introduced in the hospital in 1990, and cefepime and meropenem have been available since 1997 and 1998, respectively.

### Bacterial Isolates

We collected 199 consecutive isolates of PDRAB recovered from 72 patients colonized or infected by these organisms from January 1999 to April 2000 and from one patient with bacteremia in May in 1998. Multiple isolates from a single patient were included only if they were recovered from different body sites or recovered from the same body site more than 7 days apart. These isolates were recovered from sputum (142 isolates), wound pus (20 isolates), blood (18 isolates), bronchial washing (6 isolates), central venous catheter tips (5 isolates), pleural fluid (3 isolates), and urine (5 isolates). Thirty-three of these patients had more than one isolate (range 2 to 13 isolates) collected for this study. Four environmental isolates were recovered from a ventilator monitor board (two isolates) and tips of feeding syringe (two isolates) from an ICU. The isolates were stored at –70°C in trypticase soy broth (Difco Laboratories, Detroit, MI) supplemented with 15% glycerol before being tested.

### Antimicrobial Susceptibility Testing

MICs of antimicrobial agents for the isolates were determined by means of the agar dilution method, according to guidelines established by NCCLS (19). The following antimicrobial agents were provided by their manufacturers for use in this study: ceftazidime (GlaxoSmithKline, Greenford, UK), cefepime and amikacin (Bristol-Myers Squibb Company, Princeton, NJ), flomoxef (Shionogi & Co., Ltd. Osaka, Japan), imipenem (Merck & Co., Inc., Rahway, NJ), meropenem (Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan), ampicillin-sulbactam and trovafloxacin (Pfizer Inc., New York, NY), and ciprofloxacin and moxifloxacin (Bayer Corporation, West Haven, CN). The isolates were grown overnight on trypticase soy agar plates supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, MD) at 37°C. Bacterial inocula were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. With the use of a Steers replicator, an organism density of $10^4$ CFU/spot was spread onto the unsupplemented Mueller-Hinton agar (BBL Microbiology Systems) with various concentrations of antimicrobial agents and incubated at 35°C in ambient air.

### Time-Kill Determination

Two PDRAB isolates were tested according to methods described previously (20–22). Antibiotic combinations tested included imipenem plus amikacin, imipenem plus ciprofloxacin, imipenem plus ampicillin-sulbactam, and ciprofloxacin plus ampicillin-sulbactam. In each case, concentration of MIC and one to eight twofold dilutions lower than the MICs were tested. Viability counts were performed at 0, 2, 4, 8, and 24 hours. Synergy was defined as a decrease of $\geq$2 μg/mL in viability count of the combination at 24 h compared to that with the more active of the two agents used alone (22).

### Molecular Typing

Genotyping was determined by the random amplified polymorphic DNA (RAPD) patterns generated by APPCR and by the pulsotypes generated by PFGE. APPCR was performed with two random oligonucleotide primers: OPA-05 and OPA-02 (Operon Technologies, Inc., Alameda, CA) under conditions described previously (8). For PFGE, DNA extraction and purification were also carried out as described previously (11). DNA was digested by the restriction enzyme *Sma*I, and the restriction fragments were separated in a CHEF-DRIII unit (Bio-Rad Laboratories, Hercules, CA) at 200 V for 27 h. Interpretation of the PFGE profiles followed the description by Tenover et al. (23). PFGE profiles of the isolates were considered derived from a common ancestor (closely related isolates), if the numbers of fragment differences were three or less (23).

## Results

### Trend of CRAB and PDRAB

The rapidly increasing incidence of CRAB (from 5.88% in 1993 to 21.5% in 2000) and PDRAB (0% before 1998 to 6.5% in 2000) as causes of nosocomial infection is shown in Figure 1. This trend correlates with the increasing use of carbapenem and ciprofloxacin but not with the use of extended-spectrum cephalosporins and aminoglycosides.

### Antimicrobial Susceptibilities

All PDRAB isolates were also nonsusceptible to all of the antibiotics tested by the agar dilution method (Table 1). Most

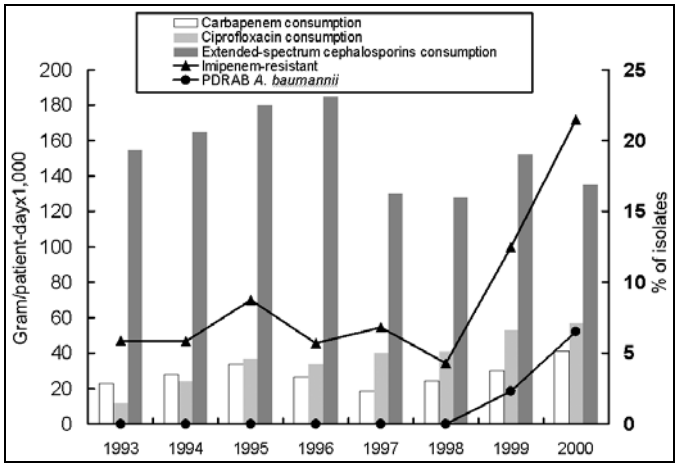


Figure 1. Annual consumption (gram/patient-day x 1,000) of carbapenems (imipenem and meropenem), extended-spectrum cephalosporins (cefotaxime, ceftroaxone, ceftazidime, and cefepime), ciprofloxacin, aminoglycosides (gentamicin, tobramycin, netilmicin, and amikacin) and percent of isolates of imipenem-resistant and pandrug-resistant *Acinetobacter baumannii* (PDRAB) at the National Taiwan University Hospital, 1993–2000.

Table 1. In vitro susceptibilities for 203 clinical isolates of pandrug-resistant *Acinetobacter baumannii* (PDRAB) determined by disk diffusion

| | MIC (μg/mL) | | | % of isolates | | |
|---|---|---|---|---|---|---|
| Antibiotic | Range | $MIC_{50}$ | $MIC_{90}$ | S | I | R |
| Ampicillin | 64–>128 | >128 | >128 | - | - | - |
| SAM | 4–>128 | 64 | 128 | 3 | 3 | 94 |
| Ceftazidime | 16–>128 | >128 | >128 | 0 | 1 | 99 |
| Cefepime | 16–>128 | >128 | >128 | 0 | 1 | 99 |
| Flomoxef | 64–>128 | >128 | >128 | - | - | - |
| TZP | 32–>128 | >128 | >128 | 0 | 4 | 96 |
| Aztreonam | 8–>128 | 64 | 128 | 1 | 1 | 98 |
| Imipenem | 8–64 | 8 | 32 | 0 | 62 | 38 |
| Meropenem | 8–128 | 16 | 128 | 0 | 8 | 92 |
| Amikacin | 32–>128 | >128 | >128 | 0 | 6 | 94 |
| Ciprofloxacin | 2–>128 | 64 | 128 | 0 | 6 | 94 |
| Trovafloxacin | 4–32 | 8 | 16 | - | - | - |
| Moxifloxacin | 2–16 | 8 | 16 | - | - | - |

TZP, piperacillin-tazobactam; SAM, ampicillin-sulbactam.

(62%) of the isolates were intermediate to imipenem, although only 8% of these isolates were intermediate to meropenem. Only 3% of these isolates were susceptible to ampicillin-sulbactam. The $MIC_{90}$ of trovafloxacin and moxifloxacin was 16 μg/mL for each.

### Synergy Tests

The results of the time-kill study of the two isolates tested—one (isolate I) that belonged to clone 5 and the other (isolate II) that belonged to clone 6 (see below)—are shown (Table 2). Only imipenem plus amikacin and imipenem plus ampicillin-sulbactam showed synergy against isolate I; synergy was detected for all four combinations for isolate II. The MICs of the two combinations with synergistic activity for isolate I remained in the resistant ranges. On the other hand, the MICs of two (imipenem plus amikacin and imipenem plus ciprofloxacin) of the four combinations for isolate II were within the susceptible ranges.

Table 2. Results of time-kill study of two isolates of pandrug-resistant *Acinetobacter baumannii* (PDRAB)

| | MIC (μg/mL)[a] | |
|---|---|---|
| | Isolate I | Isolate II |
| Antibiotic | (clone 5) | (clone 6) |
| Alone | | |
| Imipenem | 32 | 32 |
| Amikacin | 128 | 64 |
| Ciprofloxacin | 128 | 2 |
| Ampicillin-sulbactam | 128 | 32 |
| **Combination** | | |
| Imipenem + amikacin | 16/64 | 2/4 |
| Imipenem + ciprofloxacin | 32/128 | 4/0.25 |
| Imipenem + ampicillin-sulbactam | 16/64 | 8/8 |
| Ciprofloxacin + ampicillin-sulbactam | 128/128 | 1/16 |

[a]Values are the lowest concentrations (μg/mL) of each agent in combination that yielded synergy.

### PFGE and APPCR Analysis

A total of 10 PFGE profiles, pulsotypes A to J, were identified among the isolates recovered from 73 patients (Figure 2A). Pulsotype E isolates were further separated into 10 subtypes, subtypes E1 to E10 (Figure 2B). Most (46.9%) of the subtypes among the strain isolates of pulsotype E were subtype E2, followed by E3 (17.2%) and E6 (12.5%). For RAPD

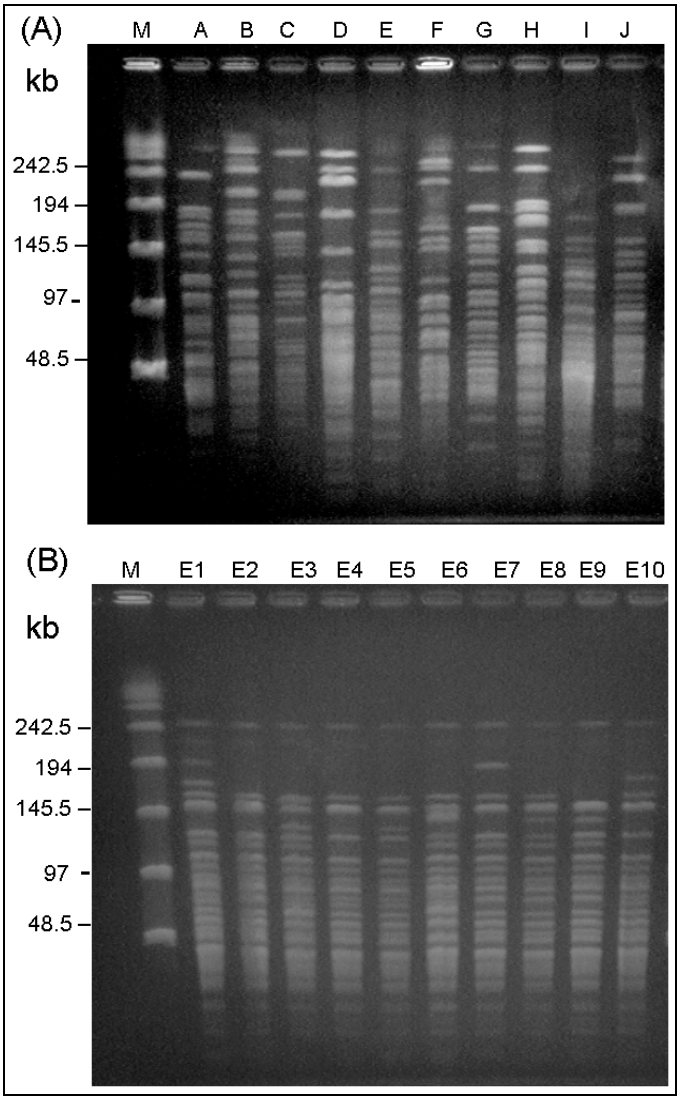


Figure 2. (A) Ten pulsotypes obtained by pulsed-field gel electrophoresis (PFGE) after digestion with *Sma*I. Lane M, molecular size marker. Lanes A to J, pandrug-resistant *Acinetobacter baumannii* (PDRAB) isolates belonging to pulsotypes A to J, respectively. (B) Ten subtypes of pulsotype E. Lanes M, molecular size marker. Lane E1 to E10, PDRAB isolates belonging to subtypes E1 to E10, respectively.

analysis using the two primers (OPA-02 and OPA-05), 10 RAPD patterns, patterns 1 to 10, were recognized (Figure 3, A and B). The 10 patterns correlated well with the 10 pulsotypes. Isolates recovered from various body sites of the same patient had identical pulsotypes and RAPD patterns.

Among the isolates, one clone (clone 5) belonging to pulsotype E and RAPD pattern 5 predominated (64 isolates) and most isolates (43, 67.2%) of this strain were recovered from ICU patients, particularly from November 1999 to April 2000 (Figure 4, A and B). The first PDRAB isolate in 1998 belonged to pulsotype A. The first clone 5 isolate was subtype E1, identified in April in an ICU (ICU-2); subtype E2 was found in another unit (ICU-7) in May. All four isolates from the equipment in ICU-4 in February 2000 belonged to clone 5 (subtype E2).

## Discussion

This report describes the trends of nosocomial infections caused by PDRAB in a university hospital and characterizes a hospitalwide epidemic due to these organisms during a 16-month period. Our results suggest three important facets. First, the upward trend in CRAB in the past 8 years and rapid emergence of PDRAB in the last 3 years are impressive. This phenomenon correlated with the level of annual use of ciprofloxacin and carbapenems in the hospital. However, risk factors for acquiring PDRAB should be studied before attributing the emergence of PDRAB clones to carbapenem and other antibiotic consumption, and before implementing an antibiotic- (particularly carbapenem) restriction program as an infection-control measure to eradicate the outbreak. Second, by using PFGE and APPCR, we demonstrated the spread of one epidemic clone in 64 of 73 patients, and nine other genotypes were observed in the outbreak PDRAB isolates. Widespread dissemination of the major clone (clone 5) in all ICUs and in most general wards of the hospital contributed to the rapid emergence of PDRAB in the hospital. Third, contrary to the findings by other investigators (20,21,24), imipenem plus amikacin, ciprofloxacin, and ampicillin-sulbactam, in the combinations tested, exhibited weak activity against the major clone (clone 5) of PDRAB. Newer fluorquinolones (trovafloxacin and moxifloxacin) also had limited potency against these PDRAB isolates.

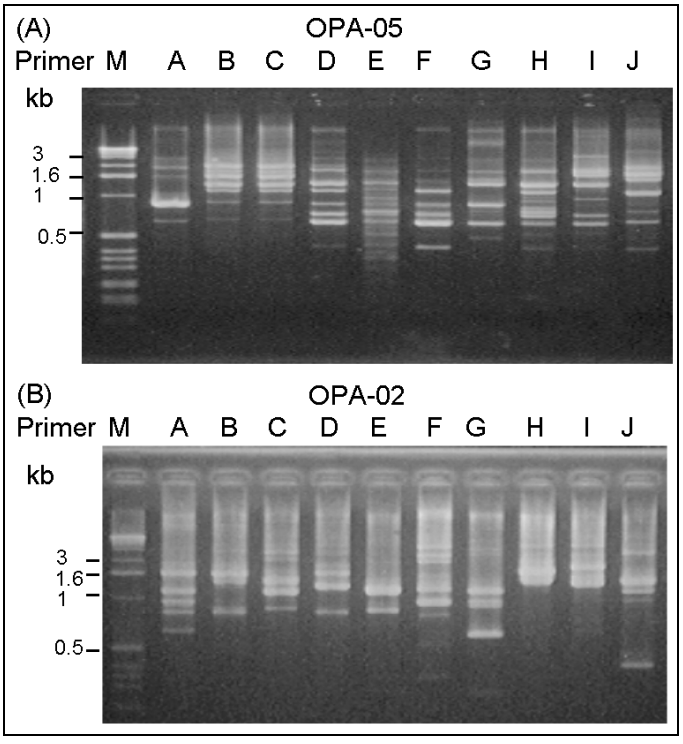


Figure 3. Random amplified polymorphic DNA (RAPD) patterns generated by arbitrarily primed polymerase chain reaction for pandrug-resistant *Acinetobacter baumannii* (PDRAB) isolates using two primers OPA-05 (A) and OPA-02 (B). Lane M, molecular size marker. Lanes A to J, RAPD patterns 1 to 10. Isolates of PDRAB belonging to pulsotypes A to J exhibit RAPD pattern 1–10, respectively.

Isolates of *A. baumannii*, particularly those recovered from patients with nosocomial infections, are frequently resistant to multiple antimicrobial agents, including cephalosporins, aminoglycosides, and quinolones (3,4,12,24). Imipenem is the most effective agent against this organism. However, with the increasing use of carbapenems and other antibiotics (such as ciprofloxacin and amikacin), particularly in institutions that have an increasing incidence of extended-spectrum β-lactamase–producing *Enterobacteiraceae* or those with hyperproduction of AmpC enzymes, the rapid and progressive emergence of CRAB and PRRAB is unavoidable (3,4,6,7,11). This phenomenon was illustrated in many countries as well as in numerous major teaching hospitals in Taiwan, including NTUH (3,4).

Different mechanisms have been involved in *A. baumannii* isolates resistant to cephalosporins and carbapenems: the altered penicillin-binding proteins, the presence of various types of β-lactamases, and the loss of porins (8,17,25). Investigation of these resistance mechanisms in our PDRAB isolates is ongoing. Although resistance emerged after considerable pressure from carbapenem use in our hospital, molecular typing approaches demonstrated that the rapid emergence of PDRAB was less likely caused by the acquisition of different resistant mechanisms by preexisting multiple clones than by the introduction of a new clone (clone 5). After detection of the first clone 5 isolate, this strain was found in many infected or colonized ICU patients as well as in patients admitted in the general wards—despite the implementation of isolation precaution and environmental surveillance. At the end of this study, the epidemic is still occurring. Further control measures such as restriction of carbapenem use (particularly in ICUs), intensification and modification of cleaning procedures for contaminated equipment, and cohorting the patients infected or colonized with CRAB or PDRAB are now being undertaken.

Previous studies of gram-negative bacilli, such as combinations of a β-lactam with amikacin, which were synergistic in vitro, have been associated with better outcomes than those achieved with nonsynergistic regimens, particularly in debilitated patients with severe infections (26). In recent reports on

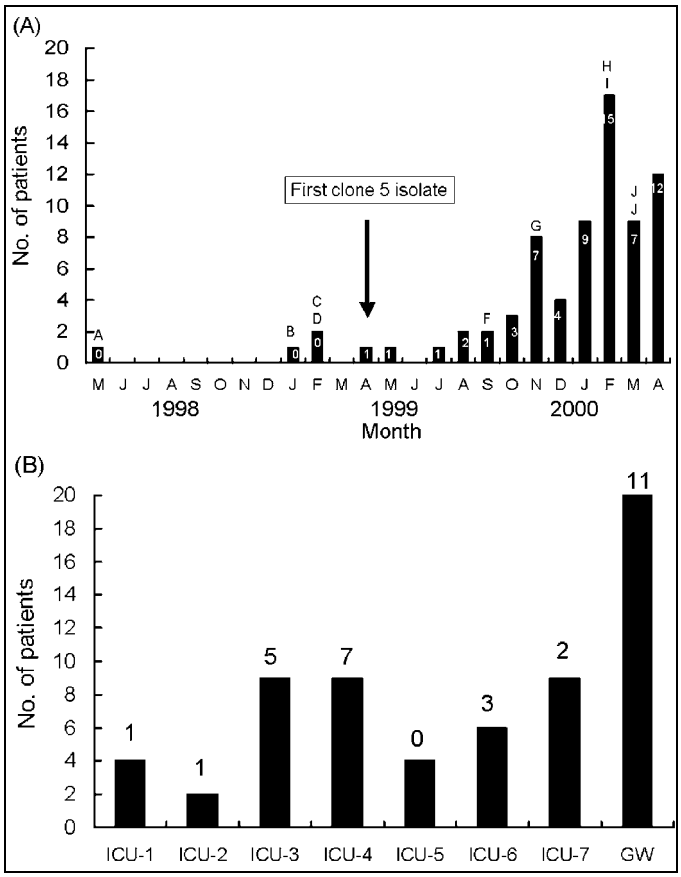


Figure 4. (A) Distribution of pulsed-field gel electrophoresis (PFGE) profiles (pulsotypes) of pandrug-resistant *Acinetobacter baumannii* (PDRAB) isolates, May 1998–April 2000. Number within each bar indicates number of isolates with pulsotype E (clone 5). Letter above indicated bar denotes isolates exhibiting pulsotype (s) other than pulsotype E. (B) Distribution of pulsotype E in seven intensive-care units (ICU-1 to ICU-7) and 13 general wards (GW). Number above each bar indicates number of isolates with pulsosubtype E2.

multidrug-resistant *A. baumannii* isolates, combinations of imipenem plus amikacin or tobramycin had better bactericidal activity against these isolates than imipenem plus sulbactam (21). Moreover, for isolates with a high MIC of amikacin (≥32 μg/mL) and ciprofloxacin (≥4 μg/mL), amikacin, or ciprofloxacin MICs in combination with in vitro synergy were not achievable clinically (20). Our study partly supports these findings. Although synergy was detected for combinations of imipenem plus amikacin and imipenem plus ampicillin-sulbactam, MICs of these agents exceeded the levels achievable in plasma, suggesting their limited potential as treatment regimens. Some of our patients with bacteremia due to clone 5 *A. baumannii* could be treated successfully with a higher dose of imipenem (3 g/day) plus amikacin. However, this regimen could not eradicate the organisms from respiratory secretions and wound pus (data not shown). Further in vitro and in vivo studies should be conducted to establish the treatment guidelines for CRAB or PDRAB infections.

In summary, we report a nosocomial outbreak due to a major clone of PDRAB in a hospital with widespread carbapenem use. This new emerging PDRAB can be considered a harbinger of the so-called post-antibiotic era. To confront the imminent threat of untreatable infection caused by this organism, a correct antibiotic strategy should be addressed, and strict compliance with basic and potential control measures for the containment of infection should be instituted.

Dr. Hsueh is an assistant professor in the Departments of Laboratory Medicine and Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine. His research interests include mechanisms of antimicrobial resistance and molecular epidemiology of emerging pathogens. He is actively involved in a national research program for antimicrobial drug resistant (Surveillance from Multicenter Antimicrobial Resistance in Taiwan-SMART).

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# Passive Antibody Administration (Immediate Immunity) as a Specific Defense against Biological Weapons

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The potential threat of biological warfare with a specific agent is proportional to the susceptibility of the population to that agent. Preventing disease after exposure to a biological agent is partially a function of the immunity of the exposed individual. The only available countermeasure that can provide immediate immunity against a biological agent is passive antibody. Unlike vaccines, which require time to induce protective immunity and depend on the host's ability to mount an immune response, passive antibody can theoretically confer protection regardless of the immune status of the host. Passive antibody therapy has substantial advantages over antimicrobial agents and other measures for postexposure prophylaxis, including low toxicity and high specific activity. Specific antibodies are active against the major agents of bioterrorism, including anthrax, smallpox, botulinum toxin, tularemia, and plague. This article proposes a biological defense initiative based on developing, producing, and stockpiling specific antibody reagents that can be used to protect the population against biological warfare threats.

Defense strategies against biological weapons include such measures as enhanced epidemiologic surveillance, vaccination, and use of antimicrobial agents, with the important caveat that the final line of defense is the immune system of the exposed individual. The potential threat of biological warfare and bioterrorism is inversely proportional to the number of immune persons in the targeted population. Thus, biological agents are potential weapons only against populations with a substantial proportion of susceptible persons. For example, smallpox virus would not be considered a useful biological weapon against a population universally immunized with vaccinia.

Vaccination can reduce the susceptibility of a population against specific threats provided that a safe vaccine exists that can induce a protective response. Unfortunately, inducing a protective response by vaccination may take longer than the time between exposure and onset of disease. Moreover, many vaccines require multiple doses to achieve a protective immune response, which would limit their usefulness in an emergency vaccination program to provide rapid prophylaxis after an attack. In fact, not all vaccine recipients mount a protective response, even after receiving the recommended immunization schedule. Persons with impaired immunity are often unable to generate effective response to vaccination, and certain vaccines may be contraindicated for them (1). For example, the vaccine against hepatitis B does not elicit an antibody response in approximately 10% of vaccines, and the percentage of nonresponders is substantially higher in immunocompromised persons (1).

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Drugs can provide protection when administered after exposure to certain agents, but none are available against many potential agents of biological warfare. Currently, no small-molecule drugs are available that prevent disease following exposure to preformed toxins. The only currently available intervention that could provide a state of immediate immunity is passive immunization with protective antibody. Passive antibody therapy was widely used in the pre-antibiotic era but was largely abandoned with the advent of antimicrobial chemotherapy (2,3). In recent years, there has been a renaissance in the use of antibodies for therapy: 10 monoclonal antibodies (MAbs) are currently licensed and dozens are in the developmental pipeline (4). This article reviews the activity of humoral immunity against several biological agents, discusses the advantages and disadvantages of an antibody-based defense strategy, and proposes stockpiling specific antibodies for use in the event of biological attacks.

## Activity of Specific Antibodies against Biological Warfare Agents

In the section below the evidence that humoral immunity is active against important biological agents is reviewed. Representative studies are cited for each pathogen.

### Anthrax

The three clinical forms of anthrax are cutaneous, gastrointestinal, and inhalational, caused by inoculation, ingestion, or inhalation of spores of *Bacillus anthracis*, respectively (reviewed in [5]). Anthrax virulence is determined by two toxins known as lethal factor (LF) and edema factor (EF). These toxins gain access to the cell through a third component known

as protective antigen (PA), which binds to the cell surface receptor (6). Vaccination studies have established a direct correlation between antibody titer to PA and survival after lethal challenge with virulent anthrax spores (7,8). Passive administration of polyclonal antibodies raised against recombinant PA is protective in mice (9) and guinea pigs (10). Animals that received immune serum providing a titer ≥1:200 were fully protected. Immune serum containing antibodies to PA can be effective in the therapy of established experimental infection in guinea pigs when given as late as 24 h after intranasal spore inoculation (11). Evidence also indicates that some antibodies bind to anthrax spore proteins and prevent their germination, suggesting a role for antibody in interfering with the early stages of infection (12).

In contrast to the unequivocal results obtained with polyclonal sera in passive protection experiments, studies with MAbs have been somewhat disappointing. A recent study evaluated the protective efficacy of four murine MAbs to anthrax toxin components (two to PA and one each to EF and LF) in guinea pigs; only one (to PA) gave partial protection, and the effect was substantially lower than that observed with polyclonal sera (10). The relative lack of efficacy of MAbs to PA relative to the protection observed with polyclonal antibody preparations may reflect a need for antibody preparations with multiple neutralizing activities.

Overall, the results indicate that passive antibody can protect against anthrax. Serum therapy was used for the treatment of human anthrax with some success in the pre-antibiotic era in uncontrolled studies (13). The Centers for Disease Control and Prevention (CDC) has recently proposed generating antibody preparations for human therapeutic use from serum of persons vaccinated for anthrax (14). The most likely mechanism of action by which antibodies to anthrax toxin proteins mediate protection is binding to toxin and impeding its interaction with the host cell. However, the process of toxin-mediated damage has many possible steps when an antibody could interfere with the process. For example, an antibody to PA could prevent this protein from binding to its cellular receptor. This mechanism of action has been validated by experiments with single-chain antibody fragments containing the antibody binding site (15). However, the relative inefficacy of single MAbs suggests that highly active antibody preparations combining MAbs of different specificities may be necessary.

### Botulinum Toxins

These toxins are produced by *Clostridium botulinum* and encompass seven antigenic types known by the letters A through G (reviewed in [16]). The different toxins are defined by specific antisera that are not cross protective. Hence, antibody to toxin A does not neutralize the other toxins. Botulinum toxins are taken up by nerve cells through pinocytosis and mediate their action by binding to neuromuscular junctions and preventing acetylcholine release leading to muscular paralysis (16). The damage to the synaptic junction is considered to be irreversible, with recovery being the result of new axonal growth that may take weeks or months. Therapy for botulism is largely supportive, although prompt administration of an antitoxin may reduce the severity of symptoms by neutralizing unbound toxin in circulation. Antitoxin therapy for botulism lowers death rates and shortens the duration of symptoms when given within 24 h of the onset of disease (17). An equine trivalent antitoxin available from CDC contains neutralizing antibodies against the most common causes of human botulism, toxin types A, B, and E. For therapy of botulism caused by other toxin types, an experimental heptavalent equine serum is available (18). Given the side effects associated with the use of equine sera, there is great interest in the generation of human antibody preparations with neutralizing activity against the seven botulinum toxins (16). Passive administration of human botulinum immune globulin derived from volunteers vaccinated with pentavalent botulinum toxoid (ABCDE) vaccine has been protective in monkeys (19) and guinea pigs (20) against aerosolized botulinum toxin.

Many neutralizing MAbs to botulinum toxins have been generated that have potential diagnostic and therapeutic applications (21–24). The epitopes recognized by certain neutralizing antibodies have been mapped to conformational antigenic determinants (25). Recent reports indicate that biological activity of botulinum toxin can be enhanced by polyclonal equine antibody binding at equimolar concentrations of immunoglobulin (Ig) G and toxin protein (26). The proposed mechanism for this effect involves a conformational change upon antibody binding to certain epitopes, which translates into enhanced toxicity in vitro at low ratios of IgG to toxin protein. Although higher ratios of antibody to toxin produce neutralization in vitro and in vivo, this observation suggests the possibility that certain antibodies to botulinum toxin can be deleterious to the host and the need for adequate amounts in therapy. Interestingly, some MAbs can transiently reverse blockage of acetylcholine release when microinjected inside ganglionic neurons (21), raising the possibility that antibodies engineered for enhanced cellular penetration may have superior therapeutic properties.

### Brucellosis

Several species of *Brucella* can cause disease in humans, including *Brucella melitensis, B. suis, B. abortus,* and *B. canis.* Antibodies specific for the O polysaccharide of *B. abortus* are protective in mice (27). When administered before infection, MAbs to the M epitope of *Brucella* spp. reduce bacterial counts in the spleens of mice (28). A panel of murine MAbs to *B. melitensis* have been shown to be effective in protecting against experimental murine brucellosis (29). Other MAbs to a common epitope in *B. melitensis* and *B. abortus* have been shown to be protective (30). For the ram pathogen *B. ovis*, antibodies to rough lipopolysaccharide and to outer membrane proteins are protective in mice (31,32). These studies indicate the existence of multiple antigens in *Brucella* spp. that can elicit protective antibody responses.

### Q Fever

*Coxiella burnetii* is the causative agent of Q fever. Relatively little recent work has been conducted on the efficacy of specific antibody against *C. burnetii* infection. However, passive transfer of antibody protective against murine experimental infection with *C. burnetii* has been reported. Protection was observed in mice given agglutinating antibodies to Phase I *C. burnetii* (33). A second study extended those findings by demonstrating that passive antibody was effective in helping to clear murine infection only if given before or at the same time as a challenge with *C. burnetii* (34). Antibody-dependent cellular cytotoxicity of *C. burnetii*–infected macrophages suggests a potential mechanism by which humoral immunity can mediate protection (35). Notably, passive antibody was not effective in T cell–deficient mice, indicating that intact cellular immunity is needed for antibody function (34).

### Plague

*Yersinia pestis* is the causative agent of plague (reviewed in [36]). Horse serum was used for treating human plague in the pre-antibiotic era, particularly in India, where prompt administration of serum was reportedly associated with reduced mortality (37). In recent years, animal studies have conclusively established that certain antibodies are protective against *Y. pestis*. Protection against experimental *Y. pestis* infection in mice vaccinated with a subunit vaccine comprising the Fraction 1 and V antigens was shown to depend on the titer of serum IgG1 (38). Passive antibody administration protects severe combined immunodeficiency (SCID) mice against lethal *Y. pestis* infection (39). Importantly, passive antibody was protective against experimental pneumonic plague (39). In mice MAbs to Fraction 1 (F1) protein of *Y. pestis* were shown to protect against bubonic and pneumonic plague (40). Interestingly, F1– variants were recovered from some MAb-treated animals, suggesting that antibody could select for variants that lacked the epitope and thus illustrating a potential problem with therapy based on a single antibody.

### Smallpox

Variola is the causative agent of smallpox (reviewed in [41]). In the early 20th century, administration of convalescent-phase sera to patients with smallpox was claimed to shorten the course of the disease and abort the pustular stage (42). A recounting of anecdotal medical experience in Hong Kong by a British medical officer stated that serum administration was effective provided that the donor had had smallpox for at least 30 days (43). Another report from India describes a patient treated with both convalescent-phase sera and vaccinia immunization who reportedly recovered faster than expected (44). The experience with the use of vaccinia virus vaccine to prevent smallpox suggests that antibody preparations could be generated that would be active against variola virus. Vaccinia immune globulin from vaccinated volunteers has been used to treat vaccinia vaccination–associated disease (45). Most importantly, administration of vaccinia immune globulin to persons in close contact with smallpox patients substantially reduced the incidence of disease compared with rates in exposed persons who did not receive passive immunization (46). Neutralizing and protective antibodies to vaccinia virus have been described that target viral envelope antigens (47). The efficacy of specific antibody in aborting or modifying the course of vaccinia and variola infection provides a rationale for using passive antibody administration to prevent smallpox in conjunction with a vaccination strategy. This strategy is supported by the fact that immune globulin has an excellent record of preventing disease when used for postexposure prophylaxis against several viral diseases, including hepatitis and varicella zoster.

### Tularemia

*Francisella tularensis* is the causative agent of tularemia (48). Horse and goat immune sera were used for therapy of human tularemia as recently as the 1940s, with efficacy reported in selected patient groups (49). Passive administration of pooled murine immune sera protected mice against 10,000 50% lethal challenge doses ($LD_{50}$ ) with the live vaccine strain (LVS) of *F. tularensis* (50). One antigen recognized by protective antibodies is bacterial lipopolysaccharide (50). The finding that antibodies to lipopolysaccharide protect against lethal challenge with LVS in mice has been confirmed, but the same antibodies are not protective against fully a virulent *F. tularensis* strain (51). Whether this finding reflects a limitation of the model used, insufficient amounts of specific antibody in immune sera, or efficacy of humoral immunity is not clear. Efficacy of passive antibody in protection against *F. tularensis* is dependent on cellular immunity, since no protection is observed in mice deficient in interferon gamma, CD4+, or CD8+ T cells (51,52). Despite the complexity of antibody action against *F. tularensis*, the observation that in certain circumstances passive antibody is protective suggests activity against this pathogen.

### Viral Encephalitides

Three viral meningoencephalitis syndromes are caused by alphaviruses: *Eastern equine encephalomyelitis virus* (EEEV), *Venezuelan equine encephalomyelitis virus* (VEEV), and *Western equine encephalomyelitis virus* (WEEV). Protective antibodies can be elicited by the alphaviruses that protect against lethal challenge in experimental murine models; one mechanism of action is interference with attachment (53,54). For EEEV, protection was associated with neutralizing and hemagglutination-inhibiting antibodies (53). For VEEV, protective antibodies have been shown to bind to a defined area of the E2 glycoprotein (55,56).

### Viral Hemorrhagic Fevers

Many viral agents are known to cause hemorrhagic fevers, including Ebola, Marburg, and Junin viruses. Passive antibody has been used for the treatment of Ebola (57), Argentine (58), and Lassa (59) hemorrhagic fevers, with encouraging results.

Furthermore, considerable evidence from animal studies indicates that passive antibody administration prevents or ameliorates disease caused by viral agents of hemorrhagic fever (60–63). Studies in mice suggest that the protective efficacy of passive antibody action against Ebola virus (EBOV) is a result of suppression of viral growth that allows development of immunity (60). Hyperimmune goat serum generated by immunization with live EBOV protected guinea pigs against lethal challenge (64). Passive antibody therapy for EBOV infection may be effective in humans, as suggested by lower death rates in recipients of blood transfusions from convalescent patients (57). Two caveats in the use of passive antibody therapy with immune sera against hemorrhagic fevers that have emerged from studies in animal models are the existence of disease-enhancing antibodies (65) and the need for high-titer sera to achieve protection (66). However, problems with deleterious antibodies and insufficient activity could potentially be avoided by the use of MAb cocktails composed only of protective antibodies with high specific activity. In this regard, MAbs to EBOV have been developed that are protective in mice even when administered 2 days after infection (67).

#### Biological Toxins

Toxin-binding antibodies have been known to be potent antitoxins since the landmark studies of Behring and Kitasato, which showed that immune sera protected against diphtheria (68). Antibody preparations continue to be used as antitoxins in the treatment of tetanus (69), diphtheria (69), botulism (18), and venomous bites (70). Specific antibodies remain the only therapeutic compounds available that are capable of neutralizing biological toxins in vivo. Hence, ample experience supports the notion that antibodies to biological toxins will protect against exposure to toxins produced by microbes used in biological warfare and may be useful for therapy of some toxin-mediated diseases.

A variety of toxins can be used for biological warfare, including ricin, trichothecene mycotoxins, and staphylococcal enterotoxins (71). MAbs to ricin have been described that protect mice against a lethal challenge with ricin toxin (72). Similarly, passive administration of MAbs to staphylococcal enterotoxin protects mice from lethal challenge with this toxin (73).

### Advantages of an Antibody-Based Defense Strategy

The above summary indicates that specific antibody can be effective against some of the major biological warfare agents. In fact, antibody preparations in the form of serum therapy were used historically for the treatment of anthrax (13), tularemia (49), and plague (37), albeit in uncontrolled trials that do not meet modern standards for establishing efficacy. The major advantage of passive antibody immunization in defense against biological weapons is that it provides a state of immediate immunity that can last for weeks and possibly months. Some human IgG isotypes have serum half-lives in excess of 30 days (74), which would confer long-lived protection to passively immunized persons. Antibodies are natural products with minimal toxicity, provided that they contain no aggregates and have no reactivity with host tissues. If vaccines are available, simultaneous administration of vaccine and antibody may be possible to provide both immediate and long-lasting protection, as is done for rabies in postexposure prophylaxis. Antibodies conjugated to enzymes, radionucleotides, or drugs could provide additional antimicrobial activities apart from those conferred by the native immunoglobulin molecule.

Although passive antibody will generally have to be given systemically, oral administration can be useful against certain gastrointestinal agents. With the exception of rabies antiserum, most antibody preparations in clinical use are given intravenously. The need for intravenous administration is a severe constraint for mass passive immunization and would likely limit this practice to a few recipients. However, this disadvantage may potentially be circumvented because Ig preparations can theoretically be administered intramuscularly. Hence, generating antibody preparations suitable for delivery into one of the large muscles of the arm or leg may be possible without the need for logistically complicated intravenous infusions. Such antibody preparations could be supplied in self-injectable devices that could allow susceptible persons to protect themselves upon notification of a biological attack. However, for this scenario to be realistic, antibody preparations with high specific activity would have to be developed that would allow administration in a small volume.

An antibody-based defense strategy against biological warfare agents can be supported by a mature technology. Antibody-based therapies were first used in the late 19th century, and more than 100 years of experience has been gained in the development of therapeutic antibodies. In the past, antibody-based therapies were dependent on immune serum that was limited in availability and was associated with substantial side effects when the serum originated from animals (2,3). In recent years, major technical advances in the ability to generate antibodies include the development of a variety of expression systems, including hybridoma, bacteria, and phage systems (75,76). Since 1997, eight MAbs have been licensed for human therapeutic use; three of these are mouse-human chimerics and five are humanized murine MAbs (4). Each of these molecules has been the product of advances in biotechnology, and their success supports the view that the technology is in place for implementing an antibody-based defense strategy.

Immunoglobulins are highly versatile effector molecules that can be adapted for use against virtually any infectious agent or toxin. In fact, antibody therapy is now available for a variety of situations in which natural antibody immunity is not likely to be effective, including prevention of re-stenosis after coronary angioplasty and the therapy for venomous animal bites, digitalis toxicity, breast cancer, and Crohn disease (reviewed in [77]). Furthermore, the fact that natural protection to a given pathogen may rely on cell-mediated immunity

does not negate the fact that passive antibody can still mediate protection. For example, protective MAbs have now been identified against such intracellular pathogens as *Ehrlichia chaffeensis* (78), *Cryptococcus neoformans* (79), *Listeria monocytogenes* (80), *Candida albicans* (81), and *Mycobacterium tuberculosis* (82), for which cell-mediated immunity is critically important for protection.

## Barriers to Developing an Antibody-Based Defensive Strategy

The use of antibody-based therapies against infectious agents in routine clinical practice is limited by several factors, including cost, need for a specific diagnosis before use, and the fact that passive immunization is more effective as prophylaxis than as therapy for established infections. Furthermore, availability of cheap antimicrobial chemotherapy for many common pathogens has reduced interest in developing antibody therapies against infectious diseases. In fact, of the 10 MAbs currently licensed for human use in the United States, only one is for an infectious disease (prophylaxis of respiratory syncytial virus infections) (4). However, these disadvantages do not necessarily apply in facing biological warfare or bioterrorism. Therapeutic immunoglobulins are undoubtedly among the most expensive drugs used in clinical practice. The high expense of Ig preparations is related to the fact that these reagents are more fragile than small molecular weight compounds and that they originate from immune donors or cell culture production and hence are costly to obtain, produce, and maintain. In addition, many of the indications for which immunoglobulins are used represent relatively small markets, and the cost efficiency associated with mass production may not apply.

One difficulty that has plagued the development of antibody-based therapies in infectious diseases is that the market size for an antibody reagent is proportional to the prevalence of disease (3). Since antibody reagents are almost always pathogen specific, the market for antibody-based therapies is often much smaller than that for drugs with broad antimicrobial activity. Small market size combined with high price and the availability of many antimicrobial drugs has not encouraged development of antibody-based therapies for many infectious diseases. However, in considering antibodies for biological defense, the market size equals the potentially vulnerable population. This consideration, combined with the fact that stockpiles would have to be replenished periodically as a result of lot expirations, could make the economic outlook more attractive to industry. Production of sufficient antibody protein for universal protection of the U.S. population against a specific biological agent would involve large-scale production and could result in cheaper unit prices.

Another problem associated with the high specificity of antibodies is that the agent would have to be identified before antibody use. However, awareness of an attack implies that the biological agent is likely to be detected once the first exposed persons become ill and a diagnosis is made. Furthermore, the number of agents likely to be employed in biological warfare or terrorism is relatively small, and it may be possible to deduce the identity of the agent rapidly. If the threat involves more than one agent, it is theoretically possible to design cocktails of immunoglobulins to protect against the likely culprits.

One aspect that has limited enthusiasm for antibody-based therapies against infectious agents is the recognition that the efficacy of an antibody is largely a function of timing of administration relative to the development of clinical symptoms. In this regard, immune sera was effective against pneumococcal pneumonia only when administered in the first 3 days after the onset of symptoms (reviewed [2,3]). For Shiga toxin-producing strains of *Escherichia coli*, the efficacy of passive antibody is largely a function of the time of administration and the dose given, with antibody efficacy declining rapidly when administered after the second day of infection (83). In fact, antibody to toxins may not be effective therapeutically once the toxin has bound to its receptor, as is the case for botulism, a condition for which late antibody therapy is relatively ineffective. However, in the event of a biological attack, the many exposed persons could likely be given antibody before the onset of symptoms. Despite reduced efficacy when administered after the onset of symptoms, antibody-based therapy is still useful for certain diseases, as evidenced by the fact that specific immunoglobulins are used for treatment of botulism (17,18), tetanus (84), Ebola hemorrhagic fever (57), and parvovirus-associated anemia in patients with AIDS (85,86).

The availability of antimicrobial therapy does not diminish the advantages of antibody-based therapies. Currently no drugs are available that specifically counteract the activity of preformed toxins, while toxin neutralization is a classical property of antibody-mediated immunity. For certain conditions, antibody therapy may have some advantages over antimicrobial therapy. For example, administration of human IgG may require only a one-time infusion, whereas antimicrobial therapy is likely to require continuous administration during the period of exposure and following infection. Furthermore, bacteria can be relatively easily engineered for resistance to antibiotic drugs. These issues were highlighted during the recent anthrax exposures, when 60 days of therapy was recommended after exposure, with a drug (e.g., ciprofloxacin) that was selected because of concerns about potential resistance in certain strains of *B. anthracis* (87). Prolonged use of antimicrobial drugs for prophylaxis against biological warfare agents such as anthrax carries inherent risks of drug toxicity and selection for drug-resistant strains among the host microbial flora (87). Antibody defense strategies can be circumvented by the generation of agents that exhibit antigenic variation. MAbs that recognize a critical domain in a microbial antigen are particularly vulnerable to the emergence of antigenic variation arising from selection during person-to-person spread or genetic engineering of the biological agent. Hence, stockpiles of MAbs can easily be made obsolete by biological agents that exhibit antigenic differences. This problem may be circum-

vented by using polyclonal antibody preparations or MAb cocktails that bind multiple epitopes in the targeted antigen. The efficacy of antibody preparations can be safeguarded by classifying the binding specificities and characteristics of antibody preparations as state secrets. Furthermore, the possibility of counterstrategies should be incorporated into the design of antibody therapeutics by specifically targeting constant epitopes that are unlikely to retain biological activity if altered. In fact, it may be possible to safeguard the usefulness of antibody preparations designed specifically for protection against biological agents by concealing their specificity in complex preparations that defy immunologic analysis.

Currently, we lack sufficient immunologic knowledge to predict the specificities and isotypes that are protective against individual pathogens. Hence, the search for protective antibodies remains empirical. Incidentally, the identification of a protective antibody de facto identifies an antigen that is capable of eliciting a protective antibody response. In the case of *C. neoformans* and *C. albicans*, MAbs to polysaccharide components were first shown to be protective and this information was used to generate conjugate vaccine that were protective in mice (88,89). Hence, a search for therapeutic MAbs can lead to an useful reagent for immediate use and also identify antigens suitable for vaccine development.

Perhaps the greatest hurdle facing the development of antibody therapies, vaccines, and new antimicrobial therapies for many agents of biological warfare is that these compounds would have to be developed without standard clinical trials. Extrapolating from observations made in animal models and in vitro is treacherous because we do not understand the correlates of protection for the overwhelming majority of infectious agents. Our state of immunologic knowledge is not sufficiently advanced to predict which antibodies or vaccines would be effective in humans. However, efficacy in animals and in vitro does mean potential efficacy in humans. Hence, in the event of an emergency it is probably better to have compounds that are effective in animal models than to have no therapies at all. In the pre-antibiotic era, the mouse pneumococcal model accurately predicted the efficacy of horse serum in humans, and the dosing of horse antipneumococcal serum was based on units derived from the mouse protection test (2).

## Polyclonal versus MAb Products

In common usage, the term polyclonal antibody preparation refers to immune sera that usually contain pathogen-specific antibodies of various isotypes and specificities. In contrast, MAb preparations consist of a single immunoglobulin type, representing one isotype with one specificity. In theory, polyclonal preparations for human therapeutic use can be generated by mixing MAbs. Each product has important advantages and disadvantages that must be weighed in considering the development of a passive antibody strategy.

Polyclonal preparations have the advantage of consisting of diverse immunoglobulins that target different antigens; the heterogeneity in isotype composition confers broader biological activity through the various constant regions. Polyclonal preparations are generally relatively easy to make, provided that immune donors are available. However, the amount of specific antibodies in a polyclonal preparation usually represents only a minute fraction of the total antibody protein. Hence, polyclonal preparations tend to have low specific activity relative to MAb preparations. For example, in a comparison of the activity of human MAbs with that of human immune globulin, 0.7 mg of a mixture of two MAbs had the same neutralizing activity as 100 mg–170 mg of tetanus immune globulin (90). Other problems associated with polyclonal preparations generated from immune donors are lot-to-lot variations in the amount of specific antibody (91), limited supply (92), and the possibility of transmission of infectious agents (93).

MAbs have the advantage that they can be defined precisely with regard to structure, specificity and activity. Furthermore, MAbs produced in vitro by hybridomas or other expression systems can provide an inexhaustible supply of immunoglobulin, thus freeing production from relying on a limited number of immune donors. However, the fact that MAbs recognize only a single epitope means that they have limited usefulness against pathogens that exhibit antigenic variation. This problem can be circumvented by generating MAb cocktails, with the caveat that such preparations would likely encounter a more complex regulatory process.

## Proposal for an Antibody-Based Defensive Strategy

Stockpiling antibody-based reagents that can be rapidly administered to exposed populations would substantially reduce the threat of many biological agents by providing a means of conferring immediate immunity to susceptible persons. For persistent threats for which vaccines are available, this measure would provide additional time for immunization, as well as reducing the threat. Development of antibody-based therapies may reduce the attractiveness of biological warfare as a weapon of terror by providing antidotes to help neutralize the threat. An aggressor could attempt to defeat a passive antibody defense by engineering the agent to express antigenic changes, proteases, or antibody-binding proteins. However, in this arms race the advantage may favor the defender, since it is technologically easier to generate a new antibody effective against the changed agent than to engineer a pathogen or agent to enhance virulence. Antigenic changes by definition create new epitopes that can be targeted by other antibodies. Antibodies can also be engineered to resist proteolysis by altering the amino acid sequence to eliminate proteolytic sites. In fact, a neutralizing antibody preparation can likely be generated much faster than new biological agents can be developed. An example of the rapidity with which therapeutic antibodies can be developed comes from the 1905 epidemic of meningococcal meningitis in New York City, when Flexner generated an effective horse antiserum within months and used it to treat patients before the epidemic abated naturally (94). Although

this example is not applicable today, given regulations on the development of therapeutics, it provides a dramatic example of the concept that antibody therapies can be developed quickly. The development of antibody-based therapies relies on technology that can respond rapidly to new threats, whereas construction of new biological agents would almost certainly require considerable basic research and development. The same may not apply to new antimicrobial chemotherapy or vaccines, which often require substantially longer development times.

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# Worldwide Occurrence of Beijing/W Strains of *Mycobacterium tuberculosis*: A Systematic Review

**Judith R. Glynn,* Jennifer Whiteley,* Pablo J. Bifani,† Kristin Kremer,‡ and Dick van Soolingen‡**

Strains of the Beijing/W genotype family of *Mycobacterium tuberculosis* have caused large outbreaks of tuberculosis, sometimes involving multidrug resistance. This genetically highly conserved family of *M. tuberculosis* strains predominates in some geographic areas. We have conducted a systematic review of the published reports on these strains to determine their worldwide distribution, spread, and association with drug resistance. Sixteen studies reported prevalence of Beijing strains defined by spoligotyping; another 10 used other definitions. Beijing strains were most prevalent in Asia but were found worldwide. Associations with drug resistance varied: in New York, Cuba, Estonia, and Vietnam, Beijing strains were strongly associated with drug resistance, but elsewhere the association was weak or absent. Although few reports have measured trends in prevalence, the ubiquity of the Beijing strains and their frequent association with outbreaks and drug resistance underline their importance.

In the early 1990s, a multidrug-resistant *Mycobacterium tuberculosis* strain was identified in New York (1). This strain, designated "W," which was associated with large institutional outbreaks of tuberculosis (TB) and many deaths, was later identified in other parts of the United States (2,3). In 1995, a large proportion of the *M. tuberculosis* strains in the Beijing area of China was reported to have mutually highly similar multi-banded IS*6110* restriction fragment-length polymorphism (RFLP) patterns; these "Beijing" strains were also present in many other populations (4).

The New York City multidrug-resistant "W" strain was, in the second half of the 1990s, recognized as a member of the "Beijing" genotype family of *M. tuberculosis* strains (5–7). The W strain is recognized by a specific IS*6110* fingerprint pattern, by multiplex polymerase chain reaction (PCR) targeted at specific insertions, or both (2,3). W family strains have IS*6110* patterns closely related to that of W, although the degree of similarity in different studies has not always been specified. Beijing strains, including the W variants, have an insertion of IS*6110* in the genomic dnaA-dnaN locus (5,7). All W family strains have a characteristic spoligotype that is shared with the whole Beijing family of strains and seems to be specific for this family (4,8,9). Spoligotyping is based on DNA polymorphism in the direct repeat region, and "Beijing" spoligotypes only contain spacers 35–43.

The combination of a widespread family of strains and, in some situations, the association with multidrug resistance has led to concern that these strains may be spreading and may have a predilection for acquiring drug resistance. Many recent studies have recorded "Beijing-like" or "W-like" strains. We have conducted a systematic review of published reports to assess how widespread the family of strains is, whether there is any evidence that it is spreading, and whether it is associated with drug resistance.

## Methods

Relevant studies were identified through computerized searches of Medline (January 1, 1990–November 1, 2001) and PubMed (January 1, 2000–November 1, 2001), manually searching key journals, searching the Internet, and cross-checking references with collections of articles on Beijing strains compiled by researchers in the field. The computerized searches used both thesaurus and free-text terms to search for tuberculosis and any of the following: molecular epidemiology, DNA fingerprinting, DNA fingerprint*, typing, type, types, restriction fragment length polymorphism, RFLP, spoligotyping, spoligotyp*, strain, and strains. The International Journal of Tuberculosis and Lung Disease, its predecessor Tuberculosis and Lung Disease, and the Journal of Clinical Microbiology were searched manually back to January 1990. A request for relevant articles was sent to all 32 participants in the European Union Concerted Action project on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis. An Internet search, using Google, used the term "Beijing strain tuberculosis." The reference lists of all included articles were searched for additional relevant studies.

Articles were included if they contained information allowing estimation of the proportion of TB patients included with the Beijing or W strains. Articles were excluded if they

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were limited to a particular outbreak, if they included only drug-resistant strains, or if <30 TB patients were included. Identified articles were subdivided into those that used spoligotyping to identify Beijing family strains and those that used other methods. Where spoligotypes were shown, estimates based on the spoligotype were used rather than any estimate given in the papers, using the proportion with spacers 35–43. Studies identifying only W strains or other W-like strains with a single IS*6110* fingerprint pattern will underestimate the prevalence of Beijing strains, since they identify only part of the family of strains. The method of patient selection was recorded when stated. In all studies, any evidence of changes over time or by age group or of any association between strain type and drug resistance was recorded.

## Results

Five thousand nineteen articles were selected from the initial search of Medline and PubMed. The titles and abstracts of these articles were scanned for relevant information, and 4,909 articles were rejected, leaving 110 articles for full text review. No further articles were identified by manual searching, but one recently published article was identified in the article collections that had not yet been indexed in the databases (10). One additional article was identified from reference list checking that was published in a Vietnamese journal not indexed by Medline, EMBASE, or Web of Science, and we have been unable to locate it. Another article was found from an Internet search, in an electronic journal (11). Of the 112 articles reviewed in full, 26 fulfilled the inclusion criteria of this review, including 16 that gave results based on spoligotyping and several that reported results from more than one area (Tables 1,2; Figure). Studies that described patients who were apparently included in other reports have been excluded (31,32).

The Beijing strain was most common in the Beijing area of China, accounting for 92% of strains (4,12). The strain was common in all the Asian studies (4,8,12–15,23–25) and also in Houston, Texas (25%), and Estonia (29%) (18,20). Some examples of the Beijing family were seen in almost all the populations studied (Tables 1 and 2).

Two studies looked at trends over time (Table 1). In China, the proportion of TB due to Beijing family strains in stored specimens going back to the 1950s was similar to the proportion among more recent specimens (12). In Gran Canaria, a dramatic increase was seen from 1992 to 1996, traced to an outbreak originating from a noncompliant patient with laryngeal TB (19). In studies over a short period, variations with age can be studied as a proxy for time trends. In Vietnam, among new cases of TB, the proportion due to Beijing strains was 71% in those <25 years of age, decreasing to 41% in those $\geq$55 years ($p < 0.001$, chi square test for trend) (14). In Bangkok, little difference was seen with age in two studies (15,24). In Hong Kong (13), Jakarta, Indonesia (8), and Estonia (18), there was no association between age and disease due to the Beijing strain. In New Jersey, among those with tuberculosis due to W-like strains, 70% of patients were <50 years old, compared with 63% of those with other strains ($p=0.2$) (9). In Gran Canaria, the median age of cases with the Beijing strain was similar to that of all cases (19). No other studies have presented results by age.

Several studies reported associations with drug resistance (Table 3). Some studies found high rates of drug resistance among Beijing strains, but others found no difference in drug resistance profiles between Beijing and the other local strains.

An association between the successful spread of Beijing strains and BCG vaccination has been suggested (4). In Jakarta, Indonesia (8), 26% of those with Beijing strains and 23% of other patients had a BCG scar. In Vietnam, although a higher proportion of those with Beijing strains than with other strains had a BCG scar, this association was no longer apparent after the data were adjusted for age (14).

## Discussion

This review has confirmed the ubiquity of the Beijing family of strains. Only a few of the smaller studies (in Martinique and French Guiana) found no examples, and the proportion of TB due to Beijing strains in several Asian studies was >50%. However, studies could only be included in the review if they mentioned the Beijing strain or strain W or presented data showing spoligotypes. Some of the excluded studies may have found Beijing strains but not reported them as such (33,34). Others may have looked for Beijing strains but not reported negative findings. The only articles identified that reported not finding Beijing strains were studies including more than one study site. It is not known how unusual it is for a genotype family of *M. tuberculosis* to be as widespread as this. Comparable data are not available for other strains, although they are beginning to be gathered, and some other strains have also been found in several distinct settings (35).

In many studies, the true proportion of TB attributable to the Beijing family of strains is hard to assess. Difficulties arise due to the variable strain definitions used and the way patients were selected for inclusion. Spoligotyping seems to be both sensitive and specific for the Beijing family and is also easily compared between studies (6). Although IS*6110* fingerprinting can also be used to detect this genotype family, with results that correlate closely with the spoligotypes, most published studies have used narrow definitions, based on a single strain or a few closely related strains defined by IS*6110* fingerprinting; such studies are thus likely to underestimate the prevalence of Beijing strains. Studies including drug resistance in the definition (2) and those that appear to have defined the strains after grouping by drug resistance (26) may also underestimate the prevalence.

Some of the studies (those in the Netherlands, New Jersey, Houston, Texas, Gran Canaria, and French Guiana and the Caribbean islands) included information on all TB patients in the population and thus provide reliable estimates of prevalence. Others were less representative, and many did not state how the patients were selected (Table 1 and 2). Studies that

Table 1. Prevalence of Beijing family strains in studies that have used spoligotyping[a]

| Reference | Setting | Yrs | Population | New TB or new + old | Prevalence Beijing strain N/N (%) |
|---|---|---|---|---|---|
| **Asia** | | | | | |
| 12 | Beijing and Hebei province, China | 1956–1960 | Stored lung biopsy samples from pneumonectomies | ? Both | 9/10 (90) |
| | | 1969–1970 | | | 8/9 (89) |
| | | 1979–1980 | | | 18/18 (100) |
| | | 1989–1990 | | | 10/12 (83) |
| | | 1956–1990 | | | 45/49 (92) |
| 4 | Beijing, China | 1992–1994 | ? Selection method | ? Both | 45/49 (92) |
| 13 | Hong Kong | 1998–1999 | Random sample | ? New | 337/500 (67) |
| 14 | Ho Chi Minh City, and Hanoi, Vietnam | 1998–1999 | ? All patients | New | 301/563 (53) |
| 15 | Bangkok, Thailand | 1999–2000 | One hospital ? Selection method | ? Both | 90/204 (44) |
| 8 | Jakarta, Indonesia | 1998–1999 | Consecutive patients one clinic | ? Both | 31/92 (34) |
| **Africa** | | | | | |
| 16 | Senegal | 1994–1995 | ? Selection method (all Beijing were relapses) | Both | 8/69 (12) |
| **Middle East** | | | | | |
| 17 | Fars Province and Tehran, Iran | 1995–1996 | All from Shiraz; ? random for others | Both | 10/97 (10) |
| **Europe** | | | | | |
| 11 | Northwest region, Russia | 1997–1998 | ? Selection method | Both | 22/100 (22) |
| 10 | Azerbaijan | 1995–1996 | Prison ? Selection method | Both | 46/65 (71) |
| 18 | Estonia | 1994 | Two hospitals, pulmonary TB | New | 61/209 (29) |
| 4 | Netherlands | 1993–1994 | Whole population | Both | 82/2,594 (3) |
| 19 | Gran Canaria, Spain | 1991–1992 | Whole island | ? Both | 0/85 (0) |
| | | 1993 | | | 10/179 (5.5) |
| | | 1994 | | | 12/148 (8.1) |
| | | 1995 | | | 18/110 (16) |
| | | 1996 | | | 35/129 (27) |
| | | 1999 | | | 9/40 (23) |
| **USA** | | | | | |
| 9 | New Jersey | 1996–1998 | Whole population | Both | 68/1,207 (6) |
| 20 | Houston, Texas | 1994–1999 | Whole population | ? Both | 326/1,283 (25) |
| **Caribbean** | | | | | |
| 21 | Cuba, outside Havana | 1994–1995 | Whole population | ? Both | 20/157 (13) |
| 22 | Guadeloupe | 1994–1996 | Whole island | ? Both | 1/95 (1) |
| 22 | Martinique | 1995–1996 | Whole island | ? Both | 0/31 (0) |
| **South America** | | | | | |
| 22 | French Guiana | 1995–1996 | Whole country | ? Both | 0/76 (0) |

[a]N/N, number with Beijing strain/ total number of patients; ?, not clear from report.

included patients from particular hospitals may be representative of an area, but referral hospitals may be biased if they accept a high proportion of drug-resistant or complex cases. Similarly, convenience samples may not be representative of the community of TB patients, particularly if the samples were kept because they were interesting in some way (e.g., drug resistant or from epidemiologically related cases). TB patients in prison (10) may not have the same strains as those in the community. Some studies included only new patients, and others included both new patients and recurrent cases. This distinction, which was often not clear in the reports, could influence the results if relapse rates differ between strains.

Table 2. Prevalence of Beijing and W-like strains in studies not based on spoligotyping[a]

| Reference | Setting | Yrs | Population | New TB or new + old | Typing methods and definitions used | Prevalence of Beijing strain N/N (%) |
|---|---|---|---|---|---|---|
| **Asia** | | | | | | |
| 23 | Henan Province, China | ? | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 59/64 (92) |
| 23 | Philippines | ? | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 34/34 (100) |
| 23 | Hanoi, Vietnam | ? | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 20/50 (40) |
| 23 | Korea | 1995 | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 99/138 (72) |
| 23 | Thailand | ? | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 31/49 (63) |
| 24 | Bangkok Nonthaburi, Thailand | 1994–1995 | Patients from 3 hospitals ? how selected. Half extrapulmonary | ? Both | RFLP + comparison with Dutch database | 80/211 (37) |
| 23 | Malaysia | ? | No information given | ? | RFLP +3.6kb *Pvu* II fragment | 17/48 (35) |
| 25 | Malaysia | 1993–1994 | Random 3% sample from whole population | ? Both | RFLP "similar" to Beijing family | 83/439 (19) |
| **Africa** | | | | | | |
| 26 | Cape Town, South Africa | 1993–1997 | Whole population | Both | RFLP "strain U", (W-like) Two closely related patterns only | 17/650 (2.6) |
| **USA** | | | | | | |
| 27 | New York City | 1992–1994 | Patients from 5 hospitals | ? Both | RFLP, strain W only | 6/302 (2.0) |
| 3 | New York City | 1990–1995 | ? selection method | ? Both | RFLP, "W-like" | 273/1,953 (14) |
| 28 | Central Los Angeles | 1994–1996 | Consecutive patients | ? Both | RFLP, strain 210 (W-related) | 43/162 (27) |
| 29 | California | 1992–1995 | All cases from specific locations | ? Both | RFLP, strain 210 (W-related) | 39/522 (7) |
| 29 | Texas | 1993–1995 | All cases from specific locations | ? Both | RFLP, strain 210 (W-related) | 16/546 (3) |
| 29 | Colorado | 1989–1994 | All cases from specific locations | ? Both | RFLP, strain 210 (W-related) | 2/256 (0.8) |
| 2 | United States (excluding NY) and Puerto Rico | 1992–1997 | All notified cases | Both | RFLP and/or PCR probe. Multidrug resistant W only | 23/104,549 (0.02) |
| **South America** | | | | | | |
| 30 | Buenaventura, Colombia | 1997–1998 | 34 treatment failure + 73 new ? selection method | Both | RFLP + PCR probe. "Similar" to W | 11/107 (10) (? 8 in new) |

[a]N/N, number with Beijing strain/total number of patient; ?, not clear from report; the different typing methods are described in the introduction. RFLP: restriction fragment length polymorphism using IS*6110*. PCR*:* Polymerase chain reaction probe is a multiplex PCR probe targeted at specific insertions. The 3.6 kb *pvu*II fragment was identified by IS*1081* fingerprinting.

In many studies, some culture-positive specimens are not typed because they are nonviable. IS*6110* RFLP typing relies on large quantities of DNA and hence on viable strains, and theoretically some genotypes may survive better than others in vitro. Spoligotyping is PCR-based so does not require viable isolates, but it is sometimes used only as a secondary method in specimens that have already been typed by IS*6110* RFLP.

Associations with drug resistance were variable (Table 3): of the 12 studies with data available, only 4 found statistically significant increases in the proportions of drug resistance among those with Beijing strains. Of the Asian studies, only one found a statistically significant increase in drug resistance in Beijing strains (14), and in Hong Kong the Beijing strains were less likely than the others to be isoniazid resistant (13). In contrast, Beijing strains were strongly associated with drug resistance in New York, Cuba, and Estonia (3,18,21). In New York, the spread of the W strain, which was mainly nosocomial and institutional, has been attributed in part to drug resistance. Once a strain has become multidrug resistant, treatment is more complicated so patients may remain infectious for a longer period. Whether the Beijing family has a particularly high probability of acquiring drug resistance is not known but is suggested by the fact that these associations with the same strain family have been found in widely distributed areas.

The published studies provided little direct evidence that the Beijing strain has been increasing. Of the two studies that included time trends, one found no increase in a population with a very high prevalence for many decades (12), and in the other the increase may be attributable to the characteristics of the index patient in the outbreak (19,36). In Vietnam, the proportion of new TB patients with the Beijing strain decreased with age, suggesting an increase in Beijing strains in the com-

Table 3. Association between Beijing family strains of *Mycobacterium tuberculosis* and drug resistance[a]

| | | Strain | | % Drug resistance | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Any | | I | | S | | MDR | | Comparison of Beijing vs. non-Beijing by drug[b] RR 95% CI[b] |
| Reference | Place, yr | Beijing | Non-Beijing | Beijing | Non-Beijing | Beijing | Non-Beijing | Beijing | Non-Beijing | Beijing | Non-Beijing | |
| 13 | Hong Kong, 1998–1999 | 310 | 181 | | | 6 | 12 | 10 | 13 | | | I 0.54 (0.30 to 0.97)<br>S 0.76 (0.46 to 1.3) |
| 14 | Ho Chi Minh City, 1998–1999 | 264 | 235 | | | 28 | 19 | 42 | 19 | 3 | 2 | I 1.5 (1.1 to 2.0)<br>S 2.2 (1.6 to 3.0)<br>MDR 1.4 (0.47 to 4.3) |
| 15 | Bangkok, 1999–2000 | 90 | 114 | | | | | | | | | No assoc |
| 8 | Jakarta, 1998–1999 | 27 | 56 | 41 | 25 | 37 | 20 | 15 | 5 | | | Any 1.6 (0.86 to 3.1)<br>I 1.9 (0.92 to 3.9)<br>S 2.8 (0.67 to 11.5) |
| 16 | Senegal, 1994–1995 | 8 | 61 | | | | | | | | | No assoc |
| 11 | NW Russia, 1997–1998 | 22 | 78 | | | | | | | 77 | 58 | MDR 1.3 (1.0 to 1.8) |
| 10 | Azerbaijan, 1995–1996 | 46 | 19 | 89 | 68 | 80 | 68 | 83 | 58 | 61 | 32 | Any 1.3 (0.94 to 1.8)<br>I 1.2 (0.84 to 1.6)<br>S 1.4 (0.95 to 2.1)<br>MDR 1.9 (0.96 to 3.9) |
| 18 | Estonia, 1994 | 61 | 148 | 70 | 14 | | | | | 34 | 2 | Any 5.0 (3.2 to 7.6)<br>MDR 17.0 (5.3 to 54.9) |
| 19 | Gran Canaria, 1991–1996 | 75 | 576 | 0 | ? | | | | | | | |
| 3 | New York, 1990–1995 | 273 (W-like) | 1,680 (not W like) | | | | | | | 93[c] | ?0 | p <0.001 |
| 21[d] | Cuba, 1994–1995 | 20 | 137 | 55–65 | 4–5 | 55–60 | 4 | 0–10 | 0.7–2 | 0 | 0.7 | Any 10.8 (4.7 to 24.5)<br>I 15.1 (5.8 to 38.9) |
| 30 | Colombia, 1997–1998 | 11 | 70 | | | | | | | 27 | 23 | MDR 1.2 (0.41 to 3.4) |

[a]I, isoniazid; S, streptomycin; MDR, multidrug resistant (at least isoniazid and rifampicin); blank spaces indicate that data are not available.
[b]Relative risks (RR) were calculated when possible from the data presented. These are shown with 95% confidence intervals.
[c] Resistant to at least four drugs. Includes 206 W strains and 40 W1 strains. Identified by RFLP, not spoligotyping.
[d]Exact numbers not clear since drug resistance data only given by strain number for IS6110 defined clusters, and two Beijing strains were not clustered. For the relative risk calculation, the minimum proportion resistant among the Beijing strains was used.

munities studied (14). No association with age was found anywhere else (8,9,13,15,18,19,24), including the two other studies restricted to new patients (13,18).

On the other hand, the ubiquity of the Beijing strain and its frequent appearance in outbreaks, particularly of drug-resistant TB, suggest that it may have the potential to spread. In Estonia, although there was no association between Beijing strains and age, TB and particularly multidrug-resistant (MDR) TB have been increasing, and most MDR TB was found to be due to Beijing strains (18). The limited amount of information available from most areas of the world and the possible biases in many of the studies make definite conclusions about the extent of spread and associations with drug resistance impossible. Through the European Concerted Action on New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis, a standard definition of the Beijing genotype is being finalized, by comparisons of large collections of strains typed with spoligotyping, IS*6110* RFLP, and Region A RFLP, which visualizes insertion of IS*6110* in the genomic dnaA-dnaN locus (ms. in preparation). Studies are planned to reanalyze available data worldwide by using standard definitions and approaches.

Further studies are also needed to include more areas in an unbiased way, to study historical specimens if possible, and to investigate the virulence (8) and transmissibility of this potentially important family of *M. tuberculosis* strains. The question to be answered is if and to what extent Beijing genotype strains have selective advantages over other *M. tuberculosis* genotypes in the ability to gain resistance and to interact with the host immune defense system. If Beijing genotype strains represent a higher level of evolutionary development of *M. tuberculosis* being selected for as a result of the introduction of tuberculostatics, which inhibit the growth of *M. tuberculosis*, then consequences for the treatment of tuberculosis will be serious.

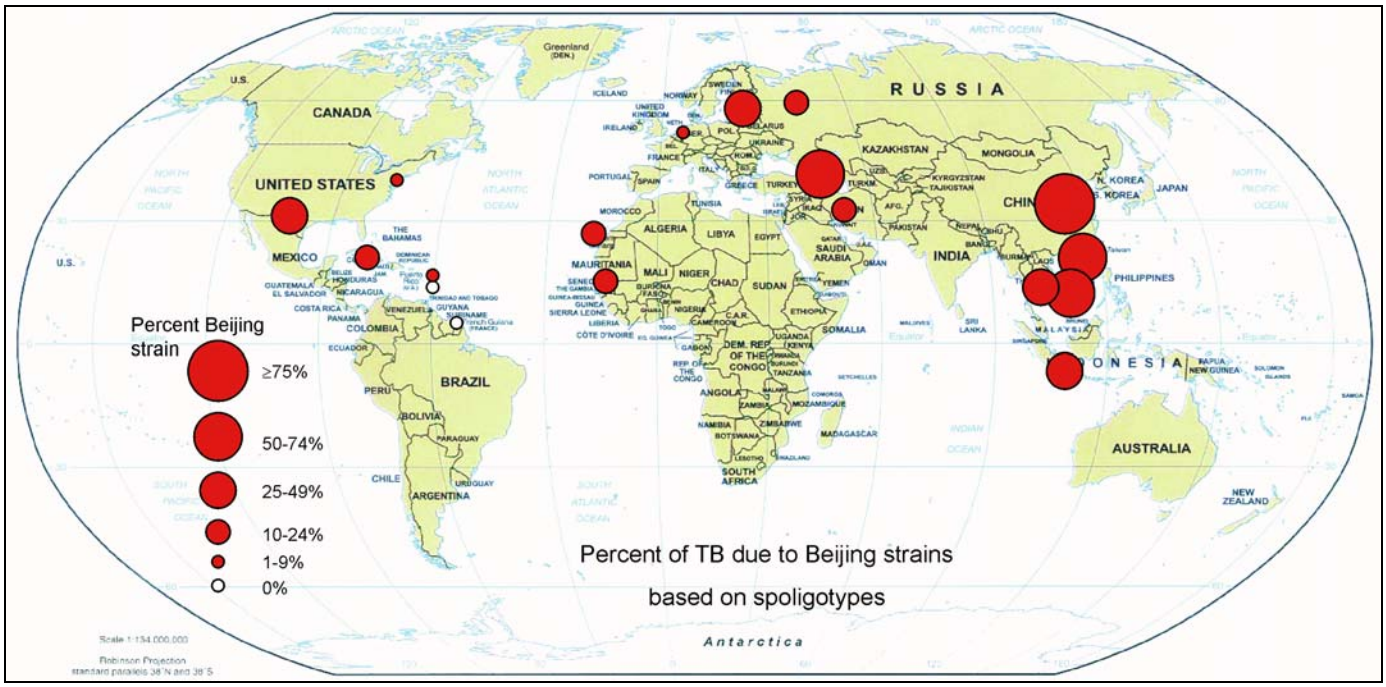


Figure. Percentage of tuberculosis due to Beijing strains. Data from studies based on spoligotyping (Table 1).

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**Synopses**. Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two.

This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

# *Haemophilus aphrophilus* Endocarditis after Tongue Piercing

**Hossein Akhondi* and Ali R. Rahimi***

Piercing invades subcutaneous areas and has a high potential for infectious complications. The number of case reports of endocarditis associated with piercing is increasing. We studied a 25-year-old man with a pierced tongue, who arrived at Memorial Health University Medical Center with fever, chills, rigors, and shortness of breath of 6 days' duration and had an aortic valvuloplasty for correction of congenital aortic stenosis.

Body piercing poses a risk for serious disease. Because it invades subcutaneous areas, piercing has a high potential for infectious complications. Such complications result from introduction of skin or mucous membrane microflora into subcutaneous tissue or from the ongoing presence of colonies of these microflora at the piercing site. Pain, edema, and prolonged bleeding may occur immediately after piercing (1), and a cyst, scar, or keloid may form at the piercing site. In various surveys, the rate of earlobe piercing infections alone has been estimated at 11% to 24%. Skin lesions or anatomic abnormalities at the site of piercing, as well as valvular heart disease, are risk factors for complications (2). Staphylococcal endocarditis of the mitral valve after nasal piercing (3), *Neisseria* endocarditis after tongue piercing (4), and *Staphylococcus epidermidis* endocarditis and mastitis following nipple piercing have been reported (5). Even though a consistent correlation is not known between piercing and endocarditis, the number of case reports is increasing, and a correlation may well exist.

Persons at high risk for complications should be treated with preventive antibiotics, just as persons at high risk for complications receive antibiotic treatment before dental procedures. The correlation between dental procedures and endocarditis has been reviewed by Van der Meer et al., who prospectively examined all cases of infective endocarditis in the Netherlands over a 2-year period (6). Of 427 patients who had been hospitalized, 64 had previous dental or other procedures in the preceding 3 months. Only 48 of the 438 patients met the qualification of having native-valve and cardiovascular anomalies that increased their risk of getting endocarditis. Using these 48 patients as study cases, the researchers found no significant difference in presence of dental procedures between patients and matched controls without endocarditis (odds ratio 1.2, 95% confidence interval 0.03 to 2.3). Two other studies (7,8) reported similar results. No study has examined the correlation between piercing and endocarditis.

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In the United States, body piercing, which is becoming increasingly common, is mainly performed by unlicenced practitioners. Only 26% of states have regulatory authority over tattooing establishments, and only six of these states exercise authority over body-piercing establishments. Piercing occurs in regulated and unregulated shops, department stores, jewelry shops, homes, or physicians' offices. Generally no antibiotic is used, and sterilization methods vary. Studies show that ear piercing can cause cephalic tetanus (a local form of tetanus caused by wounds or other head and neck infections) (8), *Pseudomonas* infections, or perichondrial auricular abscesses, especially with *Pseudomonas aeruginosa.* Tongue or oral piercing can cause Ludwig's angina (2,9,10) or may be complicated by normal oral flora, such as *Haemophilus aphrophilus*, as in this case. Genital piercing may result in *Escherichia coli* infection and may increase the risk for sexually transmitted diseases through tissue damage and exposure and unwanted pregnancy because of condom rupture (11). Systemic infections, such as toxic shock syndrome or sepsis, have also been reported (10). Among noninfectious cases, granulomatous perichondritis of the nasal ala, sarcoidlike foreign body reaction from multiple piercing, paraphimosis from a distal penis pierce, and speech impairment, together with difficulty in chewing and swallowing from oral jewelry, have been reported (1,2,9,10). Metal-associated problems include allergy (especially to nickel), eczematous rash, and lymphocytoma (2,9,10,12). We describe an incidence of *H. aphrophilus* endocarditis following tongue piercing.

## Case Report

A 25-year-old man arrived at Memorial Health University Medical Center with fever, chills, rigors, and shortness of breath of 6 days' duration. He had a history of aortic valvuloplasty at 8 years of age for correction of congenital aortic stenosis. At admission, the patient had fever of 38.9°C and a grade III/VI ejection systolic murmur accompanied by a grade II/VI diastolic blowing murmur best heard in the left sternal border area. The oral cavity was pink, and no inflammation or exudates were noticed on the pharynx. The middle portion of the tongue had been pierced, and a bispherical stud was in place (Figure). The piercing was performed 2 months before onset of illness. Extensive tattoos on the shoulders, arms, and upper torso dated back 3 years. The patient had previous dental work done but always with antibiotic prophylaxis.

Laboratory tests showed erythrocyte sedimentation rate of 41 mm/hr (normal rate**,** 0–15 mm/hr) and elevated C-reactive protein of 5.1 mg/dL (normal level 0–1). Transthoracic echocardiography was not conclusive; a transesophageal echocardiogram showed remnants of a bicuspid and deformed aortic valve with multiple vegetative lesions. Blood cultures were obtained, and the patient was started on triple antibiotics (ampicillin, nafcillin, and gentamycin). Wet preparation and acridine orange stain of the blood specimen showed gram-negative pleomorphic rods. Two of the conventional chocolate-agar cultures turned positive approximately 4 days after incu-

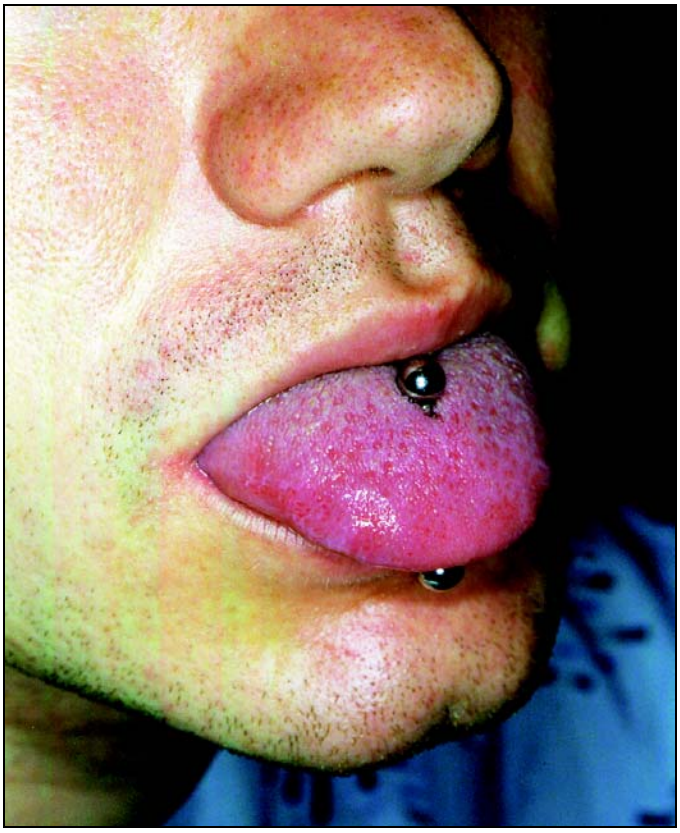

Figure. The tongue piercing of the man from this case study. The bispherical metal stud was inserted without anesthesia or preparation. Although the stud was removable, the patient had not removed it. The area around insertion was clean with no local sign of infection when the stud was removed; the tongue was not inflamed or painful.

bation and were consistent with *H. aphrophilus* (β-lactamase negative, lactose fermenting, and Mannose fermenting). The stud culture was also positive for *H. aphrophilus*. Antibiotics were modified because of sensitivity to ceftriaxone and gentamycin, and the patient was discharged to complete the 6-week course through a peripherally inserted central catheter line at home. Aortic valve replacement was recommended after completion of antibiotic therapy, but the patient did not return for treatment.

## Conclusions

Our case demonstrates *H. aphrophilus* endocarditis possibly caused by tongue piercing (or as a complication of the ongoing presence of the stud) in a patient with congenital heart disease. Colonization around the stud likely caused bacteremia and endocarditis. *H. aphrophilus* is commonly isolated from the upper respiratory tracts of humans and animals; however, its prevalence is unknown. In a previous study of piercing complications in patients with congenital heart disease (13), 43% of the study population had earlobe piercings; of these, 6% took antibiotics before piercing. Twenty-three percent of patients had piercing-related infections 1 week to 3 years after piercing. Most infections were local skin infections; no endocarditis was reported in that study.

Until prospective randomized studies shed light on the relationship between piercing and endocarditis, prophylactic measures are indicated and should be formulated, particularly for persons at high risk, e.g., those with structural heart diseases.

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# Genetic Detection and Isolation of *Crimean-Congo hemorrhagic fever virus*, Kosovo, Yugoslavia

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*Crimean-Congo hemorrhagic fever virus* (C-CHFV) strains were isolated from a fatal case and the attending physician in Kosovo, Yugoslavia. Early, rapid diagnosis of the disease was achieved by reverse transcription-polymerase chain reaction. The physician was successfully treated with oral ribavirin. These cases yielded the first genetically studied C-CHFV human isolates in the Balkans.

Crimean-Congo hemorrhagic fever (C-CHF) is a tickborne hemorrhagic fever with documented person-to-person transmission and a case-fatality rate of approximately 30%. Nosocomial outbreaks caused by *Crimean-Congo hemorrhagic fever virus* (formal abbreviation, C-CHFV; genus *Nairovirus*, family *Bunyaviridae*) have been reported in several countries. This widespread virus has been found in Africa, Asia, the Middle East, and eastern Europe. C-CHFV was first identified in the Balkans in 1954. In 1973, Gligic et al. isolated three C-CHFV strains (Ciflik 1, 6, and 11) from two *Hyalomma plumbeum plumbeum* and one castor bean tick (*Ixodes ricinus*) (1). Since then, C-CHF has occurred every year in the Balkan Peninsula, with the number of cases related to the number of ticks infected. During the spring and summer of 2001, an outbreak of C-CHF occurred in Kosovo, with 69 suspected cases, 18 of them laboratory or clinically confirmed, 6 of them fatal (2).

## Case Reports

On June 30, 2001, a 43-year-old woman (the patient with the index case), living in Kosovska Mitrovica, Kosovo, became severely ill and was admitted to the hospital with high fever, headache, nausea, generalized myalgia, and disorientation. Two days later her condition rapidly deteriorated; bleeding from the gastrointestinal tract, she was transferred to the Institute for Tropical and Infectious Diseases in Belgrade, where she died a few hours after admission.

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Blood cultures were negative for bacterial pathogens. C-CHF and hemorrhagic fever with renal syndrome (HFRS) were suspected, considering the clinical picture of the patient and the epidemiology of the diseases in this geographic area. A serum sample collected before death was tested for immunoglobulin (Ig) G and IgM specific for a variety of microorganisms (including hantaviruses, rickettsiae, and leptospira) by enzyme-linked immunosorbent assay and indirect immunofluorescent assay (IFA). No specific antibodies were detected.

To detect C-CHFV antibodies, the patient's serum was tested in twofold dilutions (initial dilution 1:8) with fluorescein-labeled goat anti-human immunoglobulin (Gibco-BRL Diagnostics, Madison, WI) on spot slides containing Vero E6 cells (ATCC CRL 1586), with approximately 50% of the cells infected with the prototype Nigerian C-CHFV strain (IbAr 10200). Titers were recorded as the greatest dilution of serum at which characteristic cytoplasmic immunofluorescence is detected. No specific antibodies were detected.

On July 6, the 38-year-old physician who had intubated the index patient when she was admitted became severely ill, with high fever, asthenia, petechiae**,** exanthema of soft palate, pneumonia symptoms, leukopenia, and thrombocytopenia (leukocyte count: $3x10^9$/L; erythrocyte count: $80x10^9$/L). No evidence of disseminated intravascular coagulation was observed.

To detect antigen in the secondary patient's serum, the immunoprecipitation procedure was used. Patient's serum sample and sera from known C-CHF–positive patients, as well as hyperimmune human gamma globulin against C-CHFV, were placed in the wells in agar plates and left overnight to diffuse; precipitin lines were formed where the concentration of the antigen and antibodies was serologically equivalent (3).

We extracted viral RNA from serum and whole blood samples from both patients. Complementary DNA was amplified by nested polymerase chain reaction (PCR) by using primers from the small (S) RNA segment of C-CHFV (4). We found a specific 260-bp band in both cases. Nucleotide sequence analysis was performed by using the OpenGene automated DNA sequencing system (Visible Genetics Inc., Toronto, Canada). As expected, the two sequences were identical. We compared the resulting sequences with those available in the GenBank database using the BLAST tool (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST/). We observed a similarity to C-CHFV S segment sequences.

Since strong evidence exists that ribavirin is effective for treating C-CHF (5,6), oral ribavirin was given to the second patient (800 mg three times on day 1, followed by 600 mg three times a day for the next 11 days). He responded successfully and, after 48 hours, became afebrile; his hematologic and biochemical parameters returned to normal. No side effects of ribavirin were present. On July 10, low titers (1:64) of IgM antibodies were detected, while on July 16, high titers (1:2,048) of IgG antibodies were detected in patient's serum samples.

The patient was placed in isolation, and all personnel wore protective clothing, such as gowns, masks, and gloves. All contact persons in the hospital were informed and advised to contact a physician if any symptoms occurred. No additional persons became ill. Samples taken from the driver and the nurse who transferred the woman from the local hospital to Belgrade were tested by IFA for the presence of antibodies to C-CHFV and were found negative, even a month after the woman's death.

Whole blood of both C-CHF cases (10% suspension in phosphate-buffered saline) was injected separately into 25-mL flasks containing Vero E6 cells. The cells were checked for C-CHFV antigen by immunofluorescence assay with hyperimmune human globulin against C-CHFV (strain Hodja). Positive cells were detected in both flasks on the 6th day after injection. The supernatant of the cells was used to infect fresh E6 cultures, and the virus was passaged six further times. We designated the virus from the fatal C-CHF case as C-CHFV/Kosovo/9553/2001, and the virus from the infected physician as C-CHFV/Kosovo/9717/2001.

Extraction of viral RNA of cell culture supernatants, amplification, and phylogenetic analysis showed that the obtained sequences were identical to each other and to the sequences amplified from the clinical samples. The nucleotide sequences were submitted to GenBank and assigned accession numbers AF428144 (for the index case) and AF428145 (for the secondary case). Phylogenetic analysis showed that these sequences clustered together with Drosdov strain (a C-CHFV strain isolated from blood of a patient in Russia), differing from it by 4%, while the genetic difference from the prototype C-CHFV Nigerian strain IbAr 10200 was 17% (Figure).

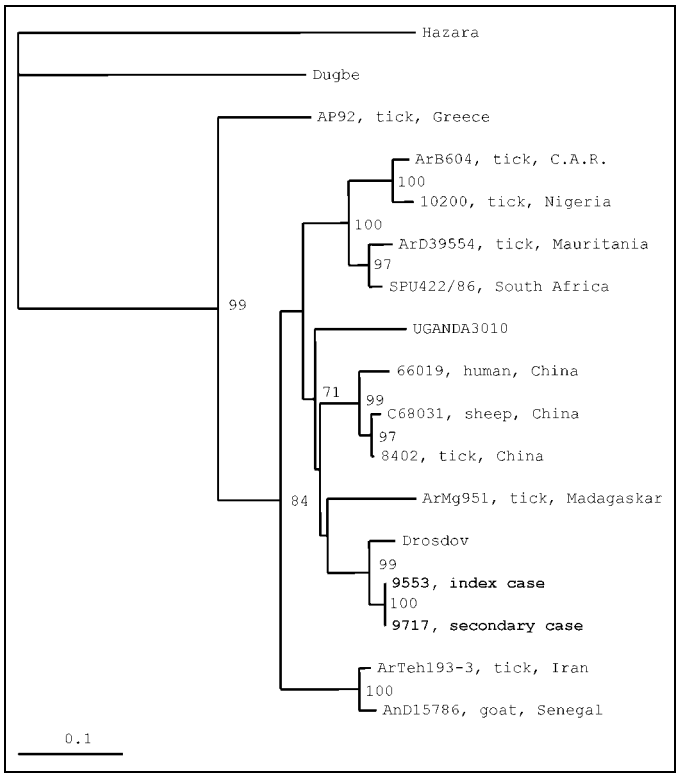


Figure. Phylogenetic relationships based on 255-nt fragment from the small RNA segment between sequences obtained from this study and respective representative Crimean-Congo hemorrhagic fever strains from GenBank. In the phylogenetic tree, sequences of other two nairoviruses, *Dugbe* and Hazara, were included; Hazara virus was used as outgroup. The numbers indicate percentage bootstrap replicates (of 100) calculated by using SEQBOOT, DNADIST, FITCH, and CONSENSE from the PHYLIP package (7); values <70% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Vertical distances are for clarity only.

## Discussion

Because of the severity of C-CHF, this illness has been difficult to study, either for diagnostic or therapeutic improvements. Antibodies to the virus are not present until 5 days after onset of illness. In addition, patients who have died of C-CHF do not usually develop a measurable antibody response. In these patients, diagnosis is achieved either by isolation of the virus from blood or tissue samples taken in the first days of illness or by molecular methods. In our first patient, who died, no antibodies were detected. Similarly, no antibodies were present during the first days of illness in the secondary case. Immunoprecipitation used for antigen detection gave the first evidence for C-CHF infection in the secondary case. However, reverse transcription (RT)-PCR established the diagnosis of the disease in both cases; sequencing the PCR amplicons confirmed the result. To our knowledge, this secondary case is the first in which PCR was used for rapid diagnosis, although use of PCR on stored acute-phase samples of infected patients has been described (4,8).

In addition, phylogenetic analysis was informative in comparing the virus strains at the genetic level, giving insights into the molecular epidemiology of the disease. The Greek C-CHFV strain (AP92), not yet associated with disease in humans, differs greatly from the Kosovo strains (24.3% nucleotide difference), forming an independent phylogenetic clade. Isolation of the virus, the standard for diagnosis, was achieved in both cases.

The first evidence of the in vitro activity of ribavirin against C-CHFV and its prophylactic use in patients' contacts occurred in 1989 (9), and the effect of oral ribavirin on outcome in C-CHF patients was described in 1995 (6). Fisher-Hoch et al. stated that, because of the low frequency of this disease and its occurrence in rural, often remote, populations, a formal drug trial is unlikely. They suggested that the only likely way to gather sufficient information on therapy was through an accumulation of reports on similar clusters as they occur (6). Ribavarin is certainly more effective in intravenous form, rather than oral; however, intravenous ribavarin is difficult to obtain. For this reason, in treating the secondary case, we used its oral form. Recovery was observed a short time after initiation of treatment, without any side effects of ribavirin.

Although the risk for fatal outcome of the secondary case was not as high, based on criteria described by Swanepoel et al. (10), we concluded that ribavirin was effective in the

patient, as he recovered rapidly after initiation of the treatment and had a successful immune response.

Concerning the origin of infection of the primary case, no details are available, such as a tick bite or any contact with C-CHF cases in the rest of Kosovo, where a C-CHF outbreak was taking place. No other C-CHF case had been reported from this region. The first patient died a few hours after admittance to the hospital, and the number of contact persons was limited. No one had any symptoms or C-CHF antibodies, except the physician who performed the intubation. He probably came in contact with patient's excreta, despite protective measures.

No tertiary case was detected, as in C-CHF nosocomial outbreaks in other countries (Pakistan, Dubai, and South Africa) (6,11–13). The virulence of the virus is likely diminished on passage, and infectivity from secondary cases is unusual (12).

Further studies are needed both in diagnostic and treatment fields to elucidate this life-threatening disease. In addition, studies at the molecular level might relate the genetic variation with the pathogenicity of the strains, rendering feasible the production of an effective vaccine.

Dr. Papa is senior scientist at the Department of Microbiology of the Aristotelian University of Thessaloniki, Greece. Her major interests focus on diagnostics and molecular epidemiology of arboviruses and retroviruses.

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# HEp-2–Adherent *Escherichia coli* Strains Associated with Acute Infantile Diarrhea, São Paulo, Brazil

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In this paired case-control study of infants with diarrhea in São Paulo, we examined the association between HEp-2–adherent *Escherichia coli* strains and diarrhea. We tested isolates from stool specimens of infants with diarrhea and matched controls in an HEp-2 cell adherence assay; we then hybridized isolates with DNA probes and identified enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). From 100 patient-control pairs, we isolated 78 HEp-2–adherent strains; of these, 61 strains were single pathogens identified in stools of infants with diarrhea. While typical EPEC was significantly associated with diarrhea ($p<0.001$), EAEC was more frequently associated with diarrhea in clinical cases (20%) compared with healthy controls (3%) ($p<0.001$). Atypical EPEC, showing a localized adherence-like pattern, was also more common in patients than controls ($p>0.1$). DAEC was isolated with equal frequency from patients and controls ($p>0.1$).

HEp-2–adherent *Escherichia coli* strains that show localized adherence (LA), aggregative adherence (AA), diffuse adherence (DA), and localized adherence-like (LAL) patterns have been implicated as diarrheal pathogens (1). In a recent study, we reported the association of HEp-2–adherent *E. coli* strains, particularly those showing LAL pattern with diarrheal stools (2). HEp-2–adherent *E. coli* strains were also identified as the most important enteric pathotype in a paired case-control study of children with diarrhea <1 year of age in São Paulo, Brazil, from May to August 1985 (3). Enteropathogenic *E. coli* (EPEC) strains were most frequently identified (23%); patients and controls did not differ in the rate of isolation of diffusely adhering *E. coli* (DAEC) (31% and 32%, respectively) or enteroaggregative *E. coli* (EAEC) (10% and 8%, respectively).

The LA shown by typical EPEC is mediated by an inducible bundle-forming pilus, which correlates with the presence of a plasmid designated the EPEC adherence factor (EAF) plasmid (4,5). EPEC strains also cause attaching and effacing lesions on eukaryotic cells that involve a 94-kDa protein encoded by the chromosomal *eae* gene (6). The pathogenicity of EPEC strains has been demonstrated in human volunteers; the role of these strains in childhood diarrhea was confirmed in epidemiologic studies (1). Atypical EPEC strains do not carry the EAF plasmid and had an LAL pattern.

Two factors, F1845 and AIDA-I, were found to encode DA in DAEC (7,8). Several recent studies have implicated DAEC strains as agents of diarrhea (9,10), while other studies have not recovered DAEC strains more frequently from diarrheal patients than from asymptomatic controls (3,11). This association may be more frequent children >2 years of age (10).

The adherence of many EAEC strains requires the presence of a plasmid with localized genes coding for AA (1); a DNA fragment from an uncharacterized region of this plasmid was described as a specific EAEC probe (12). Epidemiologic studies have implicated EAEC as a cause of diarrhea in children in developing countries, and the pathogenic potential of EAEC in human infections was substantiated by challenge studies (1).

In this study, we revisited the association between HEp-2–adherent strains and infants with diarrhea. We conducted a case-control study on *E. coli* isolates that were categorized as EPEC, EAEC, and DAEC by adherence tests and DNA probing. Our data suggest that EAEC may be a pathotype that is increasing in incidence as a cause of infantile diarrhea.

## Patients and Methods

### Patients

At the Hospital São Paulo emergency room, fecal specimens were collected from infants (children <1 year of age) with acute diarrhea lasting <5 days and from individually age-matched control infants who visited the hospital at the same time for other reasons and had not had diarrhea during the previous 30 days; specimens were collected during July – August 1999. We collected patient-control pairs for the study until we had accumulated 100 pairs in which *E. coli* was detected in stools from both the patient and the control.

### Microbiologic Studies

*E. coli* strains were isolated on MacConkey plates. Four separate lactose-fermenting colonies, presumed to be *E. coli* by colony morphology, and two non-lactose-fermenting colonies of each distinct morphologic type were cultivated in commercial test systems (Probac do Brasil, São Paulo, Brazil) for biochemical confirmation of species or genus. *E. coli* colonies were subjected to slide agglutination with polyvalent and monovalent antisera (Probac do Brasil) against O antigens of EPEC serogroups and enterohemorrhagic *E. coli*. We tested the *E. coli* colonies by adhesion assay and hybridization with DNA probes (Table 1). *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, and rotavirus were detected by standard methods (2).

All *E. coli* isolates were characterized by the pattern of adherence to HEp-2 cells in the presence of D-mannose, as

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Table 1. DNA probes identifying diarrheogenic *Escherichia coli* pathotypes[a]

| *E. coli* pathotype | Specific for | DNA probe description | Reference |
|---|---|---|---|
| ETEC | LT enterotoxin | pCVD403 (1.3-kb *Bam*HI) | 13 |
| | STp enterotoxin | pCVD426 (157-bp *Pst*I) | |
| | STh enterotoxin | pCVD427 (216-bp *Eco*RI) | |
| EIEC | Invasion | pPS55 (2.5-kb *Hind*III) | 14 |
| EHEC | Adherence | pCVD419 (3.4-kb *Hind*III) | 15 |
| | Shiga toxin 1 | pJN37-19 (1.142-kb *Bam*HI) | |
| | Shiga toxin 2 | pNN110-18 (842-bp *Sma*I-*Pst*I) | |
| EPEC | EAF plasmid | pJPN16 (1-kb *Bam*HI-*Sal*I) | 5 |
| | *eae* gene | pCVD434 (1-kb *Sal*I-*Kpn*I) | 6 |
| DAEC | *daa*C *gene* | pSLM852 (390-bp *Pst*I) | 7 |
| | AIDA-I | pIB264 (6.2-kb *Sph*I-*Cla*I) | 8 |
| EAEC | AA plasmid | pCVD432 (1-kb *Eco*RI-*Pst*I) | 12 |

[a]ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohemorrhagic *E. coli*; EPEC, enteropathogenic *E. coli*; DAEC, diffusely adherent *E. coli*; EAEC, enteroaggregative *E. coli*; EAF, EPEC adherence factor; *eae*, encoding intimin, an outer membrane protein involved in the attaching and effacing lesions promoted by EPEC; *daa*C, associated with the biogenesis of F1845, a fimbrial adhesin involved in DA; AIDA-I, protein associated with the DA phenotype; AA, aggregative adherence plasmid.

described by Scaletsky et al. (16). Monolayers were examined after 3 h of incubation. Briefly, monolayers of $10^5$ HEp-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum using 24-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ). Bacterial strains were grown in 3 mL of Tryptic Soy Broth (Difco Laboratories, Detroit, MI) for 16 h – 18 h at 37°C. Cell monolayers were infected with approximately 3 x $10^7$ bacteria (40 µL of bacterial cultures) added to 1 mL of DMEM and incubated at 37°C for 3 h. The infected monolayers were washed with sterile phosphate-buffered saline, fixed with methanol, stained with May-Grunwald-Giemsa stain, and examined under a microscope. When the adherence pattern was weak or negative, a new preparation was made and examined after a 6-h incubation period.

All *E. coli* isolates were screened by colony hybridization with DNA probes (Table 1). These probes were labeled by random primer extension kit (Rediprime II DNA Labelling System, Amersham Biosciences, Inc., Piscataway, NJ) with 50 µCi of [a-$^{32}$P]dCTP. Colony blots were hybridized at 65°C overnight, washed with 0.1X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) – 0.1% sodium dodecyl sulfate, and exposed to X-ray film overnight at -80°C.

Data derived from infants with diarrhea and from control infants were compared by a two-tailed chi-square or Fisher's exact test.

## Results

In total, we tested 402 and 430 *E. coli* colonies from 100 patients and 100 controls, respectively. We identified HEp-2–adherent strains in stool specimens from 61 infants with diarrhea. These strains were all isolated as the only pathogen; no mixed infections with a HEp-2–adherent strain and another pathogen (including enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohemorrhagic *E. coli*, *Shigella* spp., *Salmonella* spp., *Campylobacter* spp., *Y. enterocolitica*, and rotavirus) were detected in any of the cases studied. Seventeen of the controls had HEp-2 adherent isolates in their stools (Table 2). Fifty-two of the 61 adherent strains were positive for DNA sequences for EPEC, EAEC, and DAEC strains. None of the nonadherent bacteria from patients or controls were positive in the hybridization assays.

We observed four distinct patterns of adherence: LA occurred when the bacteria attached to localized areas of the HEp-2 cells in culture, forming distinct microcolonies after 3 h of incubation; DA occurred when bacteria adhered to the entire surface of the HEp-2 cells without formation of discrete microcolonies; AA was distinguished by prominent autoagglutination of the bacterial cells to each other on the surface of the cells, as well as those of glass or plastic containers; and LAL pattern, observed only in strains incubated for 6 h, was characterized by the formation of microcolonies or clusters less dense and compact than those displayed by typical LA-positive strains.

*E. coli* showing an AA pattern was more common in patients (20%) than in controls (3%) ($p<0.001$) and was detected more frequently than EPEC (17%) in patients. Nineteen (83%) of 23 *E. coli* isolates with the AA pattern hybridized with the AA probe. Of the EAEC isolates, two from patients belonged to the classic EPEC O serogroup (O44 and O78); one from a control belonged to O126.

Strains with LA were significantly associated with diarrhea (17% vs. 0%; $p<0.001$). Typical EPEC was analyzed on the basis of *eae* and EAF-positive probes; all *E. coli* isolates showing the LA pattern were hybridized with both probes. By using the *eae* DNA probe, which is specific for atypical EPEC, we found eight strains to be positive. These eight atypical

Table 2. HEp-2 adherence of *Escherichia coli* strains isolated from infants with and without diarrhea

| Adherence pattern, DNA probe[a] | No. of infected children | | p value |
|---|---|---|---|
| | Patients[b] | Controls[b] | |
| Aggregative adherence (AA) | 20 | 3 | <0.001 |
| $AA^+$ | 17 | 2 | <0.001 |
| Localized adherence (LA) | 17 | 0 | <0.001 |
| *eae*$^+$, $EAF^+$ | 17 | 0 | <0.001 |
| Localized adherence-like pattern (LAL) | 6 | 2 | 0.279 |
| *eae*$^+$ | 6 | 2 | 0.279 |
| Diffuse adherence (DA) | 18 | 12 | 0.322 |
| *daa*C$^+$ | 12 | 8 | 0.480 |

[a]*eae*, gene encoding intimin, an outer membrane protein involved in the attaching of effacing lesions promoted by enteropathogenic *E. coli*; EAF, enteropathogenic *E. coli* adherence factor; *daa*C, gene associated with the biogenesis of F1845, a fimbrial adhesin involved in DA; +, positive.
[b] n=100.

EPEC strains, which showed an LAL pattern, were isolated from six patients (6%) and two controls (2%). Of the 17 typical EPEC isolates, 15 were from classic EPEC O serogroups (4 from O55, 1 from O86, 2 from O111, and 8 from O119). Of the eight atypical EPEC isolates, seven belonged to classic serogroups (one from O26, two from O111, two from O127, and two from O128).

We found a high rate of isolation for *E. coli* that adhered with a DA pattern; however, rates were similar in patients (18%) and controls (12%). Of the 30 DAEC isolates, 20 hybridized with the *daa*C probe and none with the AIDA-I probe. Only two isolates belonged to classic serogroups (O15 and O158).

## Discussion

While EPEC has long been considered the dominant *E. coli* pathogen in São Paulo, an epidemiologic association between EAEC and acute diarrhea has not been found in Brazil until now. LA-positive EPEC has been shown to be associated with infantile diarrhea in several paired case-control studies of children <1 year of age in São Paulo (5,12). In our study, those strains were significantly more often isolated from diarrheal stools ($p<0.001$), demonstrating that EPEC continues to be an important cause of diarrheal disease in São Paulo. LA production was associated with EPEC O serogroups, as described by other researchers (2,17).

*E. coli* strains that exhibited AA and were hybridized with the AA probe were strongly associated with enteric diseases in São Paulo. Moreover, the phenotypic and genotypic approaches for the identification of EAEC strains gave almost similar results: 17/20 EAEC strains from children with diarrhea and 2/3 EAEC strains from the control group hybridized with the AA probe. In an epidemiologic study in another region of northeast Brazil, EAEC probe-positive and probe-negative strains were more likely to be associated with persistent diarrhea (18,19). Our results demonstrate that 83% of EAEC strains that were probe positive were associated with diarrhea in infants in São Paulo. We found some AA isolates belonging to the EPEC O serogroups, a finding that has been demonstrated by other authors (1). The present study is the first to show high prevalence of EAEC; in the last 25 years, EPEC strains have been prevalent in Brazil.

The pathogenic role of *E. coli* showing a DA pattern (DAEC) in the etiology of diarrheal disease is controversial (3,9–11). We found no correlation between DAEC strains and diarrhea; our results agree with those of epidemiologic studies in Australia and France (10,11), namely, that DAEC may be important diarrheal pathogens in children >1 year of age. The *daa*C probe did not show a good correlation with the DA phenotype in our study.

This study suggests for the first time that EAEC may have become a major etiologic agent of acute diarrhea in São Paulo. Further studies are needed to investigate the pathogenesis of the EAEC strains isolated in this study.

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# Infantile Pertussis Rediscovered in China

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Immunization against pertussis was introduced in China in the 1960s. Since the 1970s, no culture-confirmed pertussis cases have been reported in the country. We report six infants with culture-confirmed pertussis, who were initially diagnosed as having other respiratory diseases, at Beijing Children's Hospital, Beijing.

Immunization against pertussis was begun in China in the 1960s. Three doses of whole cell pertussis vaccine combined with diphtheria and tetanus toxoids are given at 3, 4, and 5 months of age (1). Since 1982, a booster dose has been added, given at 18–24 months of age (1).

In the 1990s, >85% of children received at least three doses of vaccine, and the incidence of pertussis (based on clinical diagnosis) remained <1/100,000 population (1). In China, pertussis is a reportable infectious disease, diagnosed by physicians. Since the 1970s, no culture-confirmed pertussis cases have been reported in the country. However, during April–June 1997, a local outbreak of pertussis was reported in a rural village (population 1,387) in southwestern China (2). A total of 285 cases were diagnosed. The ages of these patients ranged from 6 months to 80 years; 44% were <7 years of age; and 23% were 15 years of age. No deaths were reported. The suggested cause for this outbreak was relatively low vaccination coverage in the village.

The diagnosis of pertussis, especially in patients with atypical symptoms, requires clinicians' awareness and laboratory tools. To our knowledge, the current laboratory methods (e.g., culture, enzyme immunoassay serologic testing, and polymerase chain reaction assay [PCR]) are not being used to diagnose pertussis in China. In this study, we report six cases of culture-confirmed pertussis in infants seen at Beijing Children's Hospital. All six patients were initially diagnosed as having other respiratory diseases.

## The Study

To determine how much bacterial culturing would aid the diagnosis of pertussis in China, a study was conducted in a 35-bed ward for respiratory diseases at Beijing Children's Hospital from June 2000 to May 2001. This facility is the largest children's hospital in China; it has 3,000–4,000 visits daily to its outpatient department. Nasopharyngeal (NP) swabs were taken from children who had been admitted to the hospital because their cough, with or without paroxysms, had persisted for >2 weeks and was worsening. A total of 55 children (age range 35 days to 13 years) were enrolled during the study period. In addition, NP swabs were obtained from two children (ages 10 weeks and 13 years) with paroxysmal cough who were seen at the outpatient department. Information about disease history, immunization status, and cough characteristics was obtained. Physical examination, chest X-ray, and blood tests were performed. At admission, all participants were diagnosed as having bronchitis, bronchopneumonia, or pneumonia.

After collection, NP swabs were immediately spread onto charcoal agar plates supplemented with cephalexin. Details of *Bordetella pertussis* culture have been described previously (3). In brief, after inoculation, the plates were incubated in a humid atmosphere at 35°C and inspected daily for 7 days to determine pertussis-like colony growth. Suspected colonies were Gram stained and tested by slide agglutination with antisera to *B. pertussis* and *B. parapertussis* (Murex Diagnostics, Dartford, England).

Serum immunoglobulin (Ig) G antibodies to purified pertussis toxin (PT) were tested by enzyme immunoassay, as described (3). Seropositivity was determined by comparing the antibody results in patient serum samples with those in 460 healthy Chinese persons. Results exceeding the mean of the controls by two standard deviations were considered to be seropositive.

## Conclusions

Six infants <4 months of age were culture positive for *B. pertussis* (Table). Five of these patients were in the study group of 55 hospitalized children; the other was one of two children seen at the outpatient department. Before they went to the hospital, all six infants had taken broad-spectrum antibiotics but not erythromycin. None had received any doses of pertussis vaccine.

The immediate family members or other relatives of five infants (cases 1, 2, 4, 5, and 6) had concurrent and persistent cough (Table). NP swabs and serum samples were obtained from family members of cases 1, 5, and 6 (data not shown). Case-patient 1's grandmother was culture positive for *B. pertussis*. She, as well as the patient's mother and father, had been coughing for several weeks, and they all had IgG diagnostic antibodies to PT in their sera. Patient 5's mother, aunt, and 10-year-old cousin had diagnostic serum IgG antibodies to PT, and another, 8-year-old cousin was culture positive. The grandmother of case-patient 6 had been coughing for 1 month and had diagnostic serum IgG antibodies to PT.

Because antigenic divergence, with respect to PT and pertactin (PRN), has been recently found between *B. pertussis* vaccine strains and circulating strains, the PRN and PT types of eight clinical strains isolated in this study and two Chinese vaccine strains were examined. The methods used for this genotyping were LightCycler (Roche Applied Science, Mannheim, Germany) real-time PCR and fluorescence resonance

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Table. Clinical details of six infants with pertussis, China, June 2000–May 2001

| Case | Age (weeks) and sex | Duration of cough at sampling (day) | Signs and symptoms | Leukocyte count X $10^9$/L (% lymphocytes) | Diagnosis at admission | Possible source of infection |
|---|---|---|---|---|---|---|
| 1 | 5 (M) | 15 | Paroxysmal cough, cyanosis | 15.3 (62.5) | Bronchopneumonia | Mother, father, grandmother[a] |
| 2 | 9 (F) | 15 | Cough without paroxysms | 19.8 (57.7) | Bronchopneumonia | Cousin |
| 3 | 8 (M) | 15 | Paroxysmal cough | 17.6 (63.5) | Bronchitis | Not known |
| 4 | 11 (M) | 15 | Paroxysmal cough | 26.7 (52.0) | Pneumonia | Mother, father |
| 5 | 16 (M) | 20 | Paroxysmal cough, vomiting | 14.0 (55.0) | Pneumonia | Mother, aunt, cousin[a] |
| 6[b] | 10 (M) | 5 | Paroxysmal cough | 27.0 (69.9) | Bronchitis | Mother, grandmother |

[a]Culture positive for *Bordella pertussis*.
[b]Patient was treated in the outpatient department but not hospitalized.

energy transfer hybridization probes (4,5). The two vaccine strains and seven clinical strains harbored *prn1*, and one clinical strain contained *prn2*. For PT types, the vaccine strains harbored *ptxS1B* or *ptxS1D,* and all clinical strains had *ptxS1A*.

To our knowledge, this is the first report of culture-confirmed pertussis cases from China during the last 30 years. Of the 55 patients hospitalized for persistent cough, 5 (9%) were culture positive for *B. pertussis*. These results indicate that pertussis is not uncommon in the Chinese population and still causes substantial illness in infants and young children, although the pertussis vaccination coverage for the first three doses is >85% (1).

Immunity from immunization wanes with time; thus, older children and adults become susceptible to pertussis (6–8). The role of older children and adults in transmitting *B. pertussis* to unvaccinated infants has been well documented. More than 80% of hospitalized infants are not the index cases in their families (9). Our results agree with these reports. The family members and other relatives of five infants with culture-confirmed pertussis started to cough first. Evidence of laboratory-confirmed pertussis was obtained from the family members and other relatives of three infants.

Although the most serious effects from pertussis occur in young infants (10,11), all six ill infants in our study recovered. Pierce et al. reported 13 critically ill infants with confirmed pertussis, all <3 months of age (11); 4 had leukocyte counts >100 X $10^9$/L; all these infants died. Nine had leukocyte counts <100 X $10^9$/L (11). In the six ill infants in our study, the highest leukocyte count was far lower, 37 X $10^9$/L. However, our six patients had taken antibiotics before consultation.

In this study, pertussis was not initially suspected in these ill infants. None had the characteristic whoop. Pertussis at this age group is likely to be atypical, and symptoms resemble those of other respiratory tract infection, apnea, or cyanosis. Consequently, the diagnosis of pertussis is not considered and the treatment is delayed (10). These six infants were initially diagnosed as having bronchitis, bronchopneumonia, or pneumonia. The fact that pertussis was not considered in the differential diagnosis may indicate that clinicians were not aware that *B. pertussis* was circulating in the community.

Early and correct diagnosis of pertussis is important for effective therapy and prevention of transmission of the disease. Culture of *B. pertussis* from NP samples usually takes 3–7 days. In comparison with culture, PCR is a more sensitive and specific method for diagnosing pertussis (3). Measurement of specific serum antibodies to *B. pertussis* antigens by enzyme immunoassay can also facilitate this diagnosis, and results are helpful for epidemiologic studies (3,7,12). The use of culture for the diagnosis of pertussis is now being considered in Beijing Children's Hospital.

Our results suggest that a number of pertussis cases are likely being misdiagnosed in China and that the incidence of the diseases is underestimated.

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**Dispatches**. Articles should be 1,000 to 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 10), 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.

# Shigellosis Linked to Sex Venues, Australia

**Belinda O'Sullivan,* Valerie Delpech,* Giulietta Pontivivo,† Thomas Karagiannis,‡ Debbie Marriott,‡ John Harkness,‡ and Jeremy M. McAnulty***

From January 1 to July 31, 2000, 148 cases of *Shigella* infection were reported in New South Wales, Australia, compared with an annual average of 95 cases. Of reported cases, 83% were confirmed as *Shigella sonnei* biotype G infections; 80% were in homosexual men. Visiting a sex venue in the 2 weeks before onset of illness was the only factor significantly associated with shigellosis.

In 2000, a major inner-city hospital laboratory in Sydney, New South Wales (NSW), reported to local health authorities an unexpected increase in the incidence of shigellosis in homosexual men. Shigellosis outbreaks have commonly been reported related to person-to-person contact (1), child-care centers, food sources (2), institutionalized populations (3), and contaminated water (4). The infectious dose is low, with 10–100 organisms/mL sufficient for infection (5). In the United States, reports in the 1970s linked shigellosis transmission to orogenital and oral-anal sexual contact between men in bathhouses (6–7) and more recently with underlying HIV infection (8). Recent clusters of *Shigella sonnei* infection have been identified in Canada (9) and San Francisco (10) in men who have sex with men.

Sex venues in Australia are commercial establishments or bathhouses where men pay an entry fee to engage in casual sex with other men. Such establishments may provide bondage equipment, cubicles for anonymous sex, saunas, lounges, douching facilities, and toilets. At the time of the outbreak, no guidelines governed infection control in these venues.

## The Study

We contacted all public and private microbiology laboratories in inner Sydney as well as state and national reference laboratories to identify cases of shigellosis in NSW and determine the average number of cases per year. We defined outbreak-associated cases as shigellosis in homosexual men resident in NSW, aged 19–66 years, and identified by laboratories to have *Shigella sonnei* biotype G (SSBG) infection or untyped *S. sonnei* (if the laboratory did not routinely biotype *S. sonnei*) from April 1 to July 31, 2000. A patient questionnaire, piloted in 1999, included demographic details and history of illness, sexual activity, dining out, and overseas travel. Physicians from five key medical centers in inner Sydney specializing in homosexual men's health agreed to seek verbal consent from patients to either complete the questionnaire or be contacted by the investigators by telephone.

We compared reported risk exposures of patients with controls who completed the same questionnaire (all self-administered) at the same medical centers from March 1 to July 31, 1999 (Delpech, unpub. data). Controls were defined as homosexual or bisexual male residents of NSW who did not report any diarrhea in the previous 3 months.

We contacted all sex venues in inner Sydney by telephone to request permission to conduct an audit of hygiene and infection control practices. An infection control nurse inspected each venue, completing a standard audit tool that covered the appropriateness of lighting and surfaces for cleaning, cleaning regimens, hand washing, douching facilities, condom availability, and staff education. Microbiologic swabs were taken from contact surfaces including mattresses, cubicle walls, bondage equipment, door handles, and lubricant dispensers and placed in transport medium.

Aerobic cultures were performed on blood agar and MacConkey agar plates. Organisms were identified on Gram stain and routine biochemical testing. Antimicrobial susceptibility was performed by the National Committee of Clinical Laboratory Standards method with ciprofloxacin, co-trimoxazole, ampicillin, and cefotaxime. Clonality was demonstrated by using pulsed-field gel electrophoresis, enterobacterial repetitive intergenic consensus, and random-amplified polymorphic DNA polymerase chain reaction.

Univariate and multivariate logistic regression analysis was conducted by using Statistical Analytic Software (SAS; SAS Institute Inc., Cary, NC). Variables with p values $<0.25$ were applied to the multivariate model initially, and the backward stepwise elimination method was used. "Casual sexual partners" were defined by reporting "having casual sex partners in the last 3 months."

One hundred forty-eight patients with *Shigella* infection were identified from January 1 to July 31, 2000, in NSW; 123 (83%) were confirmed as having SSBG infections, compared with an annual average of 95 cases, with about 50% typed as SSBG (11). Most of the patients were reported during April and May (N=89) (Figure). Of the 123 patients with confirmed SSBG, 98 were identified as homosexual men ages 16–66 years and were defined as outbreak-associated cases. Of these, 15 (15%) were excluded because they had no physician-contact details, as these details were not routinely collected by state and national reference laboratories.

Questionnaires were completed by 42 (51%) of the remaining 83 patients; 33% of 42 questionnaires were completed by telephone interview with the investigators. Similar proportions of patients who completed a questionnaire (N=42) lived in inner Sydney (64%) compared with all outbreak-associated cases (N=148) (58%); both groups had a median age of 38 years. The main reason questionnaires were not completed was refusal to participate.

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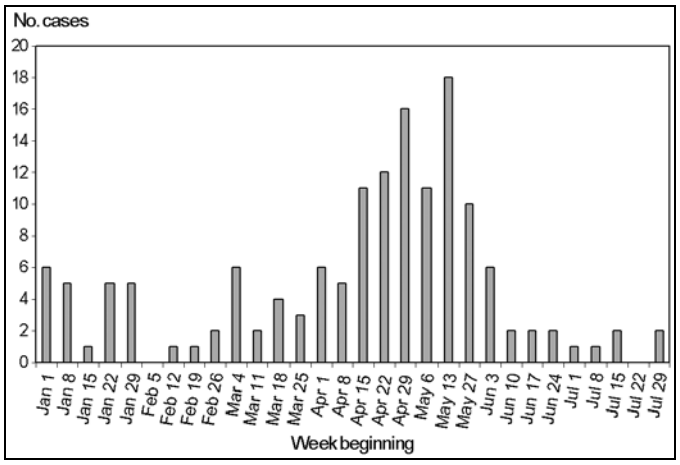


Figure. *Shigella sonnei* biotype G laboratory reports, by date of specimen collection, New South Wales, January 1–July 31, 2000.

All 42 patients reported diarrhea, 19 (45%) bloody diarrhea, 35 (83%) fever, 38 (90%) cramps, and 12 (29%) vomiting. Illness lasted a mean of 13 days (range 2–90 days), and eight (19%) were hospitalized. Twenty-two (52%) reported that they were HIV infected. HIV-infected patients were no more likely to be hospitalized (odds ratio [OR] 0.9; 95% confidence interval [CI] 0.2 to 5.3).

Sixty-five controls were included in the case-control study. Controls had a mean age of 38 years. Sixty-four percent of patients and 55% of controls resided in inner Sydney (OR 1.5; 95% CI 0.6 to 3.5). More patients (52%) than controls (48%) reported that they were infected with HIV (OR 1.2; 95% CI 0.6 to 2.6). Patients who reported HIV infections were significantly more likely to report diarrhea than those not reporting HIV infection (OR 2.9; 95% CI 1.5 to 5.9).

In univariate analysis, patients were more likely than controls to report having casual sex partners; visiting a sex venue in the previous 3 months; visiting a sex venue and having more than one sex partner in previous 2 weeks; and dining out in the last 3 days (Table). Age, either expressed as a continuous variable or in categorical 5-year age groups (OR 1.0; 95% CI 1.0 to 1.1); not always washing hands after sex in the previous 2 weeks; traveling overseas; and specific sexual behaviors or HIV status were not associated with illness. In multivariate analysis, visiting a sex venue in the previous 2 weeks was the only significant independent risk factor for shigellosis (p=0.002; OR 4.8; 95% CI 1.8 to 12.6).

All 15 identified sex-venue chains in inner Sydney were inspected during July 1 through August 1, 2000. Six (40%) of 15 had dim lighting that would prevent adequate cleaning. Ten (67%) had inadequate cleaning products and surfaces that were in a state of disrepair, including chipped, cracked, or damaged floors, wall surfaces, and furniture. Only four (27%) had a routine cleaning regimen during operational hours.

Six (40%) sex venues had no hand-washing basins, and two (13%) had basins that were inaccessible to patrons in sex activity areas. Five (33%) had anal-douching facilities, all of which sold douching tubing that was not designed for anal insertion. Only two venues with douching facilities reported routine cleaning of douching facilities after use. In one venue, fecally contaminated douching tubing was found stored in the douching room, suggesting re-use.

Seven (47%) venues offered unlimited access to condoms

Table. Characteristics of shigellosis patients and controls, New South Wales, Australia, April 1– July 31, 2000

| | Patients N=42 (%) | Controls N=65 (%) | Crude odds ratio (95% CI[a]) |
|---|---|---|---|
| Casual sex partners in the last 3 months | 37 (88) | 46 (71) | 3.1 (1.0 to 9.0)[b] |
| Visited a sex venue in the last 3 months | 31 (74) | 28 (43) | 3.6 (1.6 to 8.5)[b] |
| Visited a sex venue in the last 2 weeks | 24 (57) | 14 (22) | 4.8 (2.1 to 11.4)[b] |
| More than one sex partner in the last 2 weeks | 21 (50) | 21 (32) | 3.1 (1.3 to 7.5)[b] |
| Any sex in the 2 weeks before onset of illness[c] | 37 (88) | 52 (80) | Incalc[d] |
| Oral receptive sex in the last 2 weeks[d] | 35 (83) | 48 (74) | 1.8 (0.7 to 4.7) |
| Anal insertive sex in the last 2 weeks[d] | 26 (62) | 32 (49) | 1.7 (0.8 to 3.7) |
| Anal receptive sex in the last 2 weeks[d] | 25 (60) | 28 (43) | 1.9 ( 0.9 to 4.3) |
| Oral-anal insertive sex in the last 2 weeks[d] | 13 (31) | 22 (34) | 0.9 (0.4 to 2.0) |
| Digital insertive sex in the last 2 weeks[d] | 26 (62) | 31 (48) | 1.8 (0.8 to 3.9) |
| Not always washing hands after sex in the last 2 weeks | 16 (38) | 19 (29) | 1.4 (0.6 to 3.2) |
| Dined out at a commercial food outlet[c] in the last 3 days before onset of illness | 25 (60) | 27 (42) | 2.5 (1.1 to 5.8)[b] |
| Traveled overseas in the last 3 months | 9 (24) | 12 (18) | 1.3 (0.5 to 3.4) |
| HIV positive | 22 (52) | 31 (48) | 1.2 (0.6 to 2.6) |

[a]95% CI, 95% confidence intervals.
[b]Significant at p<0.05
[c]For controls, this question was asked in relation to previous 2 weeks rather than the 2 weeks before onset of illness.
[d]Missing values were excluded from the analysis except for sexual activity variables (e.g., oral insertive sex), for which participants were asked to indicate "yes" if they did the specified activity. As such, failure to answer these questions was considered a "no" response.

and lubricants, and eight (47%) dispensed one condom and lubricant sachet on entry. Only two venues reported that staff members received infection control education.

A total of 63 microbiologic swabs were taken from 11 venues. No *Shigella* species were isolated, but 18 (29%) cultures, including 6 of 12 sites from one venue, grew coliform bacteria, indicating fecal contamination. Eight (57%) of 14 mattress swabs from different venues grew coliforms. Environmental organisms were isolated from 36 (57%) swabs. Ninety percent of case isolates were resistant to ampicillin and cotrimoxazole, and 98% showed a similar pattern of clonality.

Given the variability of standard infection-control practices across sex venues, *Shigella* may have been transmitted either directly during casual sex or indirectly from contact with contaminated surfaces or douching equipment. While visiting a sex venue was the only significant risk factor associated with shigellosis, 40% of patients reported not having attended a sex venue. Other factors that we did not measure may have led to transmission in these persons, including casual or sexual contact with other people with shigellosis outside sex venues, contact with fomites, or eating contaminated food. The foodborne route is unlikely, as this outbreak did not affect the general community.

Despite active surveillance, some underreporting of cases is likely in this outbreak because not all patients would have consulted a physician or had a fecal specimen obtained. However, the rate of physician visits for shigellosis is likely to be higher than for other less severe diarrheal illnesses (12). While the use of historical controls makes evaluating food- and waterborne risk factors for shigellosis difficult, we believe that the evaluation of sexual behaviors in homosexuals is likely to be reliable as they show little variation over the study period (13).

An interagency approach was used to develop and conduct plans to control the outbreak. Actions included a health promotion campaign focused on homosexual men; a shigellosis forum attended by owners, managers, and cleaners of sex venues; and the interagency development of infection control guidelines for such establishments. Guidelines for infection control should be followed and equipment and surfaces in sex venues should be cleaned regularly in adequate lighting. Patrons should have easy access to and be encouraged to use hand-washing facilities to minimize the likelihood of transmission of enteric pathogens. Homosexual men should routinely be given information about the ongoing risk of transmission of enteric pathogens.

**Acknowledgments**

We thank the physicians, local public health and local government authorities, microbiology laboratories, the AIDS Council of NSW, and sex venue owners and managers for their work on this outbreak.

Ms. O'Sullivan has a masters in public health from the University of Sydney and has worked in a range of public health settings as part of the New South Wales Public Health Officer Training Program coordinated by the NSW Health Department. Her interests are in epidemiology, research, and health promotion.

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# Human Infection Caused by *Leptospira fainei*

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We report a human case of leptospirosis in which the spirochete was detected by dark-field microscopy examination of cerebrospinal fluid (CSF) and isolated from both CSF and blood. *Leptospira fainei* was identified by sequencing the 16S rDNA gene, which had been amplified by polymerase chain reaction. This case confirms the role of *L. fainei* as a human pathogen and extends its distribution to southern Europe.

Leptospirosis is a worldwide zoonosis, usually transmitted to humans through contaminated water or direct exposure to the urine of infected animals. The clinical spectrum of the disease ranges from an influenza-like syndrome to Weil's disease and multiple organ dysfunction syndrome (1). The causative agents of human leptospirosis belong to the genus *Leptospira,* which contains both saprophytic and pathogenic species (1). *L. fainei* was first isolated from pigs in Australia (2). Subsequently, several reports based on serologic testing suggested that *L. fainei* might be pathogenic for humans as well (3,4). This potential has been recently confirmed by isolating *L. fainei* from the urine of two patients and the blood of one patient in Denmark (5). We report a typical case of leptospirosis in which *L. fainei* was directly observed in the cerebral spinal fluid (CSF) and was isolated from both CSF and blood.

## Case Report

In February 2001, a 39-year-old man was hospitalized in Marseilles, France, with fever and disorientation. This truck driver lived in Portugal and had been driving in Spain for 2 weeks before entering France. He arrived in France 3 days before admission. The day of admission, his co-workers found him confused and febrile after sleeping in his truck. He had no previous relevant medical history. On admission, his temperature was 40°C with tachycardia, and his blood pressure was 120/80 mm Hg. Clinical examination showed a drowsy, confused man with a bilateral headache, provoked myalgia of the legs, hepatalgia, and conjunctivitis. Neurologic examination showed signs of meningeal irritation, including cervical rigidity, Brudzinski's sign, hyperesthesia, and photophobia. There was no rash, and the rest of the physical examination was normal. Results of biochemical investigation included increased alanine aminotransferase (61 IU/L), aspartate aminotransferase (78 IU/L), lactate dehydrogenase (781 IU/L), fibrinogen level (6.44 g/L), and C-reactive protein (273 mg/L), associated with hypoglycemia (2.9 mmol/L), hypoalbuminemia (26 g/L), hypoproteinemia (54 g/L), and hypocholesterolemia (2.3 mmol/L). The leukocyte count was 11,000/mm$^3$ with 69% polymorphonuclear forms. Thrombocytopenia (110 G/L) was also observed. The kaolin cephalin time was 58 s (control 34 s), and the prothrombin rate was 42%. Blood smears showed no parasites. Cerebral scanning was normal. Analysis of the CSF on day 1 showed 4 leukocytes/mm$^3$, 50,000 erythrocytes/mm$^3$, and elevated protein levels (1.48 g/L). On day 2, another CSF analysis showed 75 leukocytes/mm$^3$, with 70% polymorphonuclear forms, 5,500 erythrocytes/mm$^3$, and increased protein (0.64 g/L). Direct examination by dark-field microscopy of the CSF from day 2 was performed and controlled by two experimental investigators, who observed many spirochetes (6). The patient received a 10-day treatment with 12 g/day intravenous amoxicillin. The fever decreased to 38°C on day 4 and resolved on day 7. The patient was discharged from the hospital and remained well. No occupational or recreational risks for leptospirosis could be established. The patient had been traveling recently in Spain and Portugal but had no apparent exposure to sources of leptospires.

## The Study

Specific diagnostic tools were used to identify the spirochete responsible for this infection. Single 0.1 mL- and 0.01 mL-aliquots of lithium-heparin anticoagulated whole blood were spread onto 10 mL of *Leptospira* medium, a polysorbate medium similar to Ellinghausen and McCullough modified Johnson and Harris medium (EMJH) (Bio-Rad Laboratories, Inc., Aulnay/Bois, France), and was incubated at 30°C. The same procedure was carried out for CSF. For urine samples, the specimens were first filtered on 0.45- and then 0.22-µm filters before inoculation. Once a week, 10 µL of culture medium was examined by dark-field microscopy. Cultures from both blood and CSF were positive and yielded *Leptospira* after 1 week of incubation. Urine cultures remained negative. No agglutination of the isolated strain was obtained with any reference sera, except a weak agglutination (titer 400) with serovar hurstbridge antiserum. The genomic DNA of the spirochete was extracted from the blood culture by the Qiamp blood kit procedure (QIAGEN GmbH, Hilden, Germany). For polymerase chain reaction (PCR), universal 16S rDNA primers fD1 and rP2 (7) were used, and sequences of the PCR products were obtained as previously described (8). The 16S rDNA sequence of the isolate had 100% identity with the prototype strain sequence of *L. fainei* (GenBank accession no. 60594) (Figure 1). Pulsed-field gel electrophoresis of NotI macrorestriction fragments of Leptospira DNAs was performed as previously described (9). However, the NotI macrorestriction pattern was quite different from all patterns previously recorded for a large collection of Leptospira strains belonging to different species and serovars (Figure 2). Sera collected on days 4, 8, 10, and 45 were tested by a microim-

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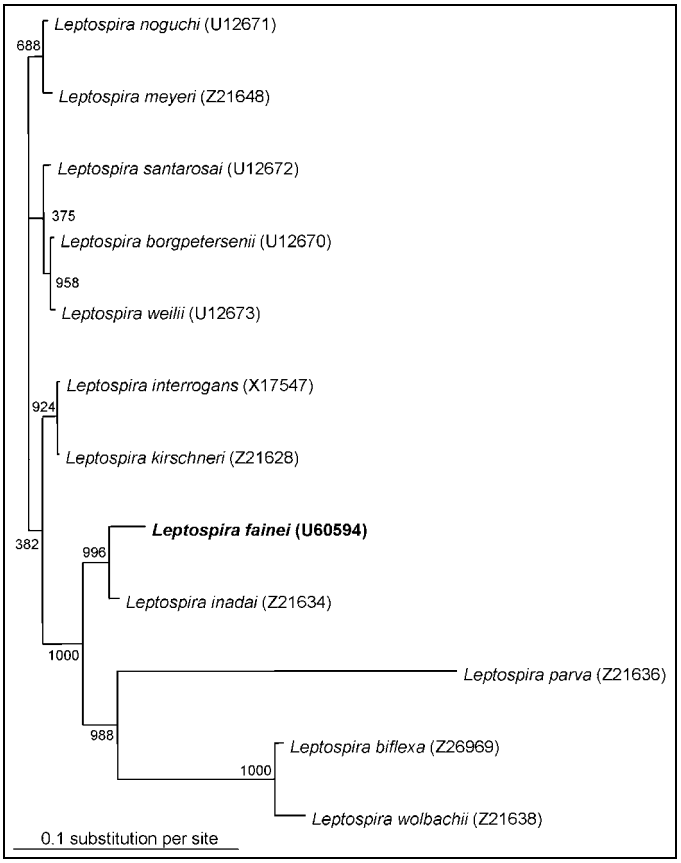


Figure 1. Dendogram representing phylogenetic relationships among members of the genus *Leptospira*. The tree was derived from a 1,295-bp fragment of the 16S rRNA gene and was constructed by the neighbor-joining method. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching point. GenBank accession numbers are given in parentheses.

munofluorescent antibody assay (MIFA) with *L. biflexa* serovar patoc, *L. fainei, L. interrogans,* and own strain as antigens (cut-off titer _200); Western immunoblot with *L. biflexa* patoc and *L. fainei* as antigens; and a microagglutination test (MAT) (10,11). For MAT, sera were incubated with suspensions of live leptospires belonging to 17 distinct serogroups, including a strain of *L. fainei* serovar hurstbridge. The titer was defined as the highest dilution giving 50% agglutination. The presence of immunoglobulin (Ig) M was investigated by an enzyme-linked immunosorbent assay (ELISA) developed at the Institut Pasteur, with serovar patoc as an antigen (12). All sera were negative by MIFA, MAT, and IgM ELISA, even when *L. fainei* was used as an antigen. For Western blot, the spirochetes were grown on *Leptospira* medium centrifuged and used at a concentration of 2 mg/mL. After blocking, the nitrocellulose was incubated overnight at 4°C with patient sera diluted 1:50. Seroconversion was demonstrated by Western blot analysis. The first two sera were negative by Western blot, but a reaction with *L. fainei* was observed in IgG and IgM at days 10 and 45. Sera reacted with protein bands of 28, 25, 24, and 19 kDa from *L. fainei*, while no reaction was obtained with serovar patoc (Figure 3).

## Conclusions

This clinical picture was highly suggestive of leptospirosis, with the association of meningeal syndrome, provoked leg myalgias, and conjunctivitis; nonspecific laboratory findings included hepatic enzyme elevation, hyperleukocytosis, thrombocytopenia, and low prothrombin rate (1). In fact, the case was considered clinically to be leptospirosis. The isolated *Leptospira* was identified as closely related to *L. fainei* on the basis of 16rRNA amplification and sequencing. The strain has been isolated in our laboratory in Marseille, where this species had never been cultivated. Thus, laboratory contamination is unlikely, and the isolated strain of *L. fainei,* which is close to

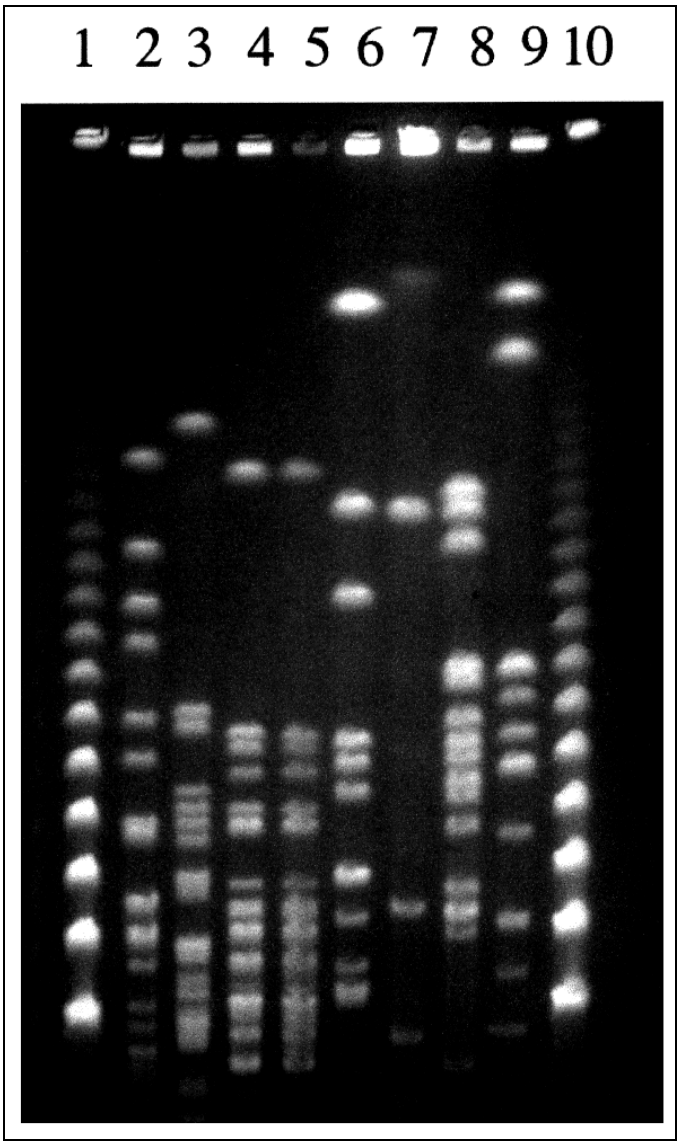


Figure 2. *Not*I restriction patterns of *Leptospira* strains obtained with the Bio-Rad apparatus (Richmond, CA) with a pulse time ramped from 5 to 90 s for 36 h. The lanes contain lambda concatemers (lanes 1 and 10) and DNA from isolates: patient strain (lane 2*)*; *L. fainei* hurstbridge, strain But 6 (lane 3); *L. inadai* Lyme, strain 10 (lane 4); *L. inadai* biflexa, strain LT430 (lane 5); *L. biflexa* patoc, strain Patoc I (lane 6); *L. meyeri* semaranga, strain VS173 (lane 7); *L. kirschneri* grippotyphosa, strain MoskvaV (lane 8); and *L. interrogans* icterohaemorrhagiae, strain Verdun (lane 9).

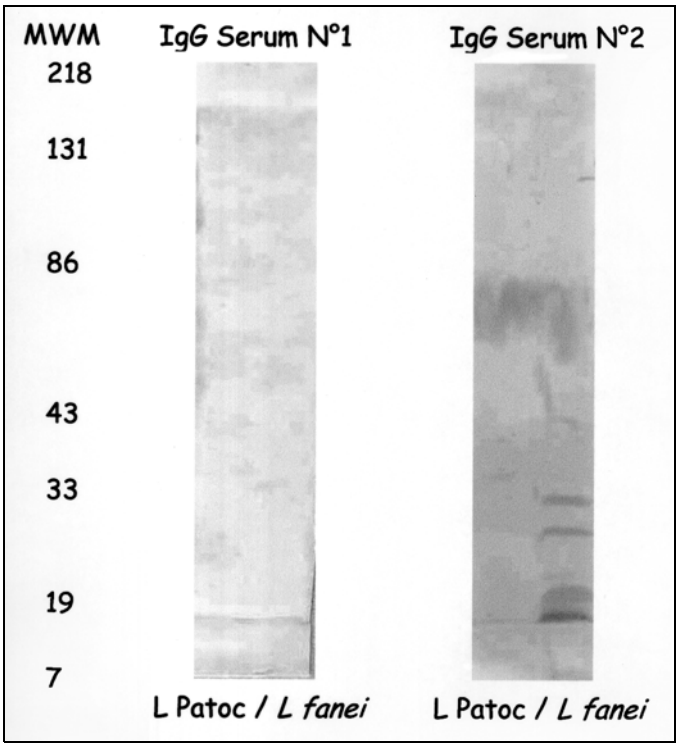


Figure 3 . Western immunoblot of the patient's acute- (No. 1) and convalescent-phase (No. 2) sera on *Leptospira* serovar patoc and *L. fainei.* MWM indicates molecular weight markers.

the serovar hurstbridge, can be considered the causative agent of the patient's meningitis. Moreover, a spirochete resembling a leptospire was seen by well-trained investigators at the dark-field microscope.

*L. fainei* has been only recently been considered an emerging pathogen. In Australia, a sample of 723 human sera from patients from a dairy and pig-producing area of Victoria, all of whom had symptoms consistent with leptospirosis, was submitted for leptospirosis serologic testing. The sera were also tested for antibodies to *L. fainei* serovar hurstbridge. MAT titers $\geq$128 were detected in 13.4% (3). Furthermore, a leptospirosis surveillance program was conducted for 12 months on the entire population of the Seychelles; the incidence of leptospirosis was 10/100,000, with a 20 % seroprevalence of *L. fainei*. This organism was suspected to be involved in severe forms such as acute renal failure, pulmonary hemorrhage, and possibly death (4).

The first human isolation of *L. fainei* occurred in Denmark, from two patients (5). The first patient had chronic disease with increasing jaundice for 6 months before admission to the hospital, and test results showed elevated hepatic enzymes. The second patient had abdominal and lower back pain for 5 months and severe headaches and dizziness for 2 months before admission. Thus, both patients had atypical chronic disease, unlike the typical case of leptospirosis that we describe.

In our case, we tested sera 4, 8, 10, and 45 days after onset of illness. No reactivity was detected by MIF and MAT tests, although a reaction in IgG and IgM at day 10 and 45 was detected by Western blot. Early antibiotic treatment may have affected the serologic responses. The two Danish patients from whom *L. fainei* was isolated were tested by MAT and showed no substantial serologic reaction (5). However, published serologic studies have shown that an immunologic response may be observed, in some cases at a high level (4). As reported here, Western blot may prove to be a good tool for diagnosis. Antibiotic treatment by amoxicillin (12 g/day for 10 days) was effective. In the Seychelles, for 8 patients among 75 with confirmed leptospirosis, a 5-day treatment with penicillin was insufficient to eradicate the bacteria, as indicated by positive PCR results (4). Treatment for longer than 1 week may be necessary, because *L. fainei* could be still isolated from Danish patients after a 1-week treatment with intravenous penicillin, but not after 4 weeks of amoxicillin treatment (3).

This case confirms the pathogenic role of *L. fainei* in humans and extends its geographic distribution to southern Europe. The clinical finding of our case did not differ from that of other *Leptospira* infections, but the route of exposure remains unknown. Although the usual procedures of direct detection, isolation, and identification of leptospires are effective for *L. fainei,* we did not observe substantial serologic reactivity. Further studies, including Western blot, are needed to explain the weak immune response and to evaluate the prevalence of infection by this newly discovered *Leptospira* species.

**Acknowledgments**

We thank Odessa Deffenbaugh for linguistic assistance.

Dr. Arzouni is a physician in the Clinical Microbiology Laboratory of the University Hospital Timone, Marseilles, France. He is especially interested in spirochete infections and has developed techniques for molecular detection and cultivation of spirochetes in human specimens.

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# Nature of the Virus Associated with Endemic Balkan Nephropathy

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Endemic Balkan nephropathy (EBN), a disease restricted to three Balkan countries (Bulgaria, Rumania, and Yugoslavia), is characterized by a progressive shrinking of the kidneys and, in some cases, tumors in the proximal regions of the urinary tract (1,2). A coronavirus was reported to be involved in the etiology of the disease, mostly on the basis of the isolation of a virus in cultures of kidney cells from a patient with EBN (1,3). In addition, EBN-associated virus is reported to share serologic homology with human coronaviruses OC43 and 229E, as well as the porcine transmissible gastroenteritis coronavirus (formal name: *Transmissible gastroenteritis virus* [TGEV]), a virus that our laboratory has been studying for 16 years (4,5). The objective of this commentary is to clarify whether the EBN-associated virus is in fact related to members of the *Coronaviridae* family (6).

## Characterization of the Virus in EBN Primary Kidney Cell Cultures

The EBN-associated virus was isolated from primary kidney cells cultures, grown from fresh renal biopsy specimens of clinically confirmed cases of EBN (3). The virus grown in the primary kidney cultures was used to infect Vero cells (ATCC CRL 1586) and sent to our laboratory for further identification.

A titration method was set up for the EBN-associated virus in Vero cells, as described for coronaviruses (7). The virus had a small plaque phenotype and titers of $10^6$ to $10^7$ PFU/mL. No specific neutralization was observed when polyvalent or monoclonal antibodies that neutralized TGEV or the human coronaviruses OC43 or 229E were used in a standard neutralization assay. Furthermore, we observed no reactivity by immunofluorescence microscopy with the same antisera and specific monoclonal antibodies (7) on cells infected with the EBN-associated virus. In contrast, cell cultures infected with human coronaviruses or TGEV were positive with the corresponding antibodies.

Since coronavirus morphology is easily recognized by electron microscopy, Vero cells infected with the EBN-associated virus were embedded in resin for electron microscopy, and ultrathin sections were examined. Coronaviruses interacting with the cell membrane or inside the cell cytoplasm were easily seen when grown in swine testicle cells (Figure, A and C). Coronaviruses bud at the intermediate compartment and Golgi membranes (Figure, C) and the cytopathic effect on infected cells differed from that observed after infection by the EBN-associated virus. The most characteristic features of EBN-associated virus infection were the accumulation of stacked intracellular membranes and a general disorganization of the cytoplasmic membranous system (Figure, D). No apparent effect on the nucleus structure was observed. Electron-dense spherical virus particles approximately 30 nm in diameter were observed in the cytoplasm of infected cells but not in the nucleus. The size of these particles corresponds to that of the virions partially purified from the same cells. The particles

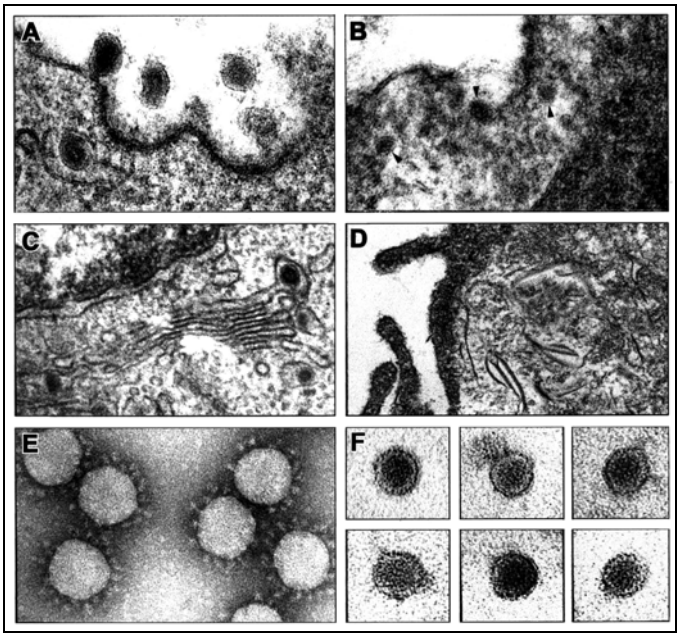


Figure. Electron microscopy images of thin sections and partially purified virions from cells infected with coronavirus or a virus tentatively associated with the endemic Balkan nephropathy (EBN). A and C. Electron microscopy images of thin sections of swine testicle cell infected with porcine transmissible gastroenteritis coronavirus (TGEV), showing virus binding to cell membrane at 8 h postinfection (A) or immature TGEV virions in the Golgi cisternae (C). B and D. Micrographs of thin sections of Vero cells infected with the virus tentatively associated with EBN at 12 h postinfection. B. The presence of EBN virions in the cytoplasm of the infected cells is indicated by arrows. D. Disorganization of the cytoplasmic membranous system in EBN-infected Vero cells. Electron microscopy images of concentrated TGEV (E) or EBN (F) virions negatively stained with 2% uranyl acetate. Bars in panels A–F represent 50 nm.

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appear to have an internal dense nucleocapsid (Figure, B). No viral factories were identified in association with the membranous structures. No viruslike particles were observed in uninfected Vero cells that resembled the virions described in the infected ones.

Supernatants of Vero cells infected with the EBN-associated virus were concentrated 100-fold by ultracentrifugation and visualized by negative staining with 2% uranyl acetate. Using purified TGEV as a standard, we observed only one type of spherical virion with a homogeneous mean virion size 28.4 nm ± 2 nm in diameter (coefficient of variation 7.1%; n=30) (Figure, F). In contrast, electron microscopy preparations of TGEV observed in parallel showed virions approximately 120 nm in diameter, with a corona of typical projecting peplomers (8,9) (Figure, C). The morphology of TGEV clearly differed from that of the EBN-associated virus that had no peplomers. The EBN-associated virion morphology and size were similar to that of small nonenveloped viruses such as picornavirus and parvovirus.

## Conclusion

The virus source used in these experiments is the same as that previously analyzed (3), and led to the tentative conclusion that a coronavirus was present in the primary cell cultures from patients with the endemic nephropathy. The dominant and only virus detected in the cell cultures infected with the EBN-associated virus was unrelated to coronaviruses. Accordingly, we think that the involvement of a coronavirus should no longer be considered in EBN induction. Further studies are needed to clarify the nature of the 28.4-nm, non-enveloped virus particles found in the kidney cells of patients with EBN and to determine whether this virus is the causal agent of the disease.

**Commentary**. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.


## Acknowledgments

We thank Dusko Vasic and the staff of the Department of Urology, District Hospital, Doboj, Bosnia, for providing the kidney biopsy specimens.


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# Jet-Black Eschar

**Janet R. Gilsdorf**
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At first glance, the baby appeared healthy. She was pudgy, pink-cheeked and impeccably clean, and rested quietly in the mother's arms—until we disturbed her; then she cried with the weary wail of an infant hurting for days.

"This one came first," said the mother, pointing to a quarter-sized red, swollen nodule to the right of the baby's spine. Embedded in its center was a jet-black eschar the size of a pea. I prodded the edge of the nodule; the baby curled her legs, buried her head in the mother's shirt, and screamed. "There are more," continued the mother, a pretty young woman with an anxious expression, pointing at the other nodules—one on the occiput, one on the labia majora, one on the left upper chest. Two of these also had black eschars.

Our first questions were routine and probably anticipated by the mother: When did the fever start? How high had the fever been? When did the lumps appear? What medicines had the baby received? The mother carefully answered each question, underscoring her responses with layers of detail, struggling to remain composed. As she spoke, each word, each gesture, each facial expression carried an air of earnestness, reinforcing her belief that the answers would help make the baby well again. While the mother gently rocked back and forth in the examining-room chair, the infant clung to her chest as fiercely as a wide-eyed baby lemur clings to his mother's furry belly.

The questions that followed were more circumspect and, to the mother, probably puzzling and far removed from the immediate problem of her feverish baby with the lumps. Still, she answered each one completely, dragging information out of her memory as if it were buried treasure. How much did the baby weigh at birth? What was her medical history? Had anyone at home been ill? Where had she traveled?

We were ready to zero in on possible diagnoses. The next set of questions must have seemed truly absurd to the mother. Another person, one not so engrossed in the well-being of the baby, might have thought our inquiries weird or intrusive, or even trivial or irrelevant. Do you have any spiders at your house? Have you seen bugs crawling on the baby? Has she been in a hot tub? Are you and the baby's father blood relatives (first or second cousins)? How old was the baby when the umbilical cord fell off?

Then, I reached for the bioterrorism protocol from the state health department, which prompted a new set of questions. Has the baby been in contact with imported rugs or animal hides? Has she been in contact with anyone who works on a ranch or with livestock? And finally the question we didn't ask until last winter: Has the baby been in contact with anyone (grandparents, family friends, babysitters) who works at a mail-sorting facility? The mother's face tensed with bewilderment and disbelief. But, again, she answered carefully and thoughtfully, persevering as we filed through our differential diagnoses.

A biopsy of the neck nodule was sent for culture and histologic testing. The baby was admitted to the pediatric ward and was started on piperacillin/tazobactam and gentamicin treatment. The next day, the pathology report came back. The lesion was consistent with ecthyma gangrenosum. Large numbers of bacilli were observed, none with the morphologic features of anthrax. The culture report confirmed *Pseudomonas aeruginosa.*

Dr. Gilsdorf is professor of pediatrics at the Medical School and professor of epidemiology at the School of Public Health, University of Michigan, and director, Pediatric Infectious Diseases, C.S. Mott Children's Hospital, Ann Arbor, Michigan. Her research laboratory studies both the molecular epidemiology of *Haemophilus influenzae* colonization and disease and pathogenic factors of *H. influenzae*, focusing on identifying virulence genes associated with acute otitis media and on defining the structural, functional, and immunologic parameters of adherence pili.

**ANOTHER DIMENSION.** Submit 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 invoke 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.

# Polymerase Chain Reaction for Screening Blood Donors at Risk for Malaria: Safe and Useful?

**To the Editor:** Transfusion-transmitted malaria, although extremely uncommon in most countries not endemic for malaria, may have fatal consequences if undetected (1,2). Benito and Rubio (3) addressed this timely issue by presenting data on screening of blood donors at risk for malaria in Spain with a seminested polymerase chain reaction (SNM-PCR). Of 125 donors at risk (immigrants from malarious areas), these researchers identified five cases of *Plasmodium falciparum* by using SNM-PCR with a 5-mL EDTA blood sample. Benito and Rubio's conclusion was that the SNM-PCR could serve as the reference test for screening blood (3). Obviously, this conclusion implies the potential use of blood donations from donors who were at risk but whose PCR results were negative. We believe that this practice would be dangerous and could lead to the administration of unsafe blood.

The PCR, like any method based on direct detection of the parasite, does have a shortcoming: the amount of specimen processed determines the limit of detection. Even if the described PCR method detected a single parasite in the 5-mL blood sample used by the authors (3) (hypothetical sensitivity 0.0002 parasites/μL), a standard 450-mL blood donation could still contain ≤90 parasites and have a negative PCR result. However, with the "best" sensitivity reported by Benito and Rubio (3) of 0.004 parasites/μL, a standard 450-mL blood donation could contain <1,800 parasites and still be tested negative by SNM-PCR. Surely, <1,800 parasites is enough to cause disease in a blood recipient. As few as 10 parasites per donation (perhaps even fewer) may cause disease. Theoretically, any method would have to detect a single parasite per unit of blood to be safe, thus requiring a hypothetical detection limit of $2.2 \times 10^{-6}$ parasites/μL. However, a sample equal to the unit of donated blood (450 mL) would have to be processed to achieve this level. In addition, one would have to assume that parasites are equally distributed in the peripheral blood at the time of donation.

Little is known about the frequency of very low parasitemias. No large-scale epidemiologic studies have been conducted in which large amounts of blood (e.g., the equivalent of a blood donation or 450 mL) were collected. The authors (3) could not confirm the PCR-positive cases by microscopy. This finding suggests very low levels of parasitemia, below the sensitivity of thick smears, in the range of 1–20 parasites per μL (4). Similar results were observed in blood donors associated with transfusion-transmitted malaria in the United States, in which malaria smears were positive in only 17 of 49 donors (1). PCR is similar to microscopy in screening donors at risk, even if the detection limits are different. Hommel and Gilles report in Topley and Wilson that for disease-endemic countries "the use of PCR… …is not, despite its much increased sensitivity, a complete guarantee of safe blood, because the absence of parasites in a 20-μL sample does not exclude the possibility of infection in the remaining volume of the 450 mL blood unit" (5).

On the other hand, one might argue that screening the whole blood supply for malaria by PCR may detect the rare blood donation with undetected malaria, with higher parasitemias. However, the generally accepted deferral criteria for blood donors at risk seem highly efficient. In the United States, only 14 cases of transfusion-transmitted malaria were reported from 1990 through 1999 (1). The same authors estimate that this deferral policy led to 50,000 rejected donations in a total of 13 million per year (0.3%). At an estimated expense of $2.00–$3.00 per PCR, a general screening program would cost more than $20 million–$30 million per year. Each case of malaria prevented would therefore cost in excess of several million U.S. dollars.

Several novel diagnostic methods have been developed recently (6). However, we agree with Mungai et al. (1). These methods, including PCR, have still not been shown to detect the lowest possible parasitemia that can cause malaria. Showing that a method is able to detect donors at risk for malaria, as done by Benito and Rubio (3), is insufficient. On the contrary, the only convincing study design would be to show that donors at risk who have a negative PCR result also do not harbor parasites and cannot transmit the disease. Accordingly, careful screening of blood donors in nondisease-endemic countries, in accordance with the established exclusion criteria, remains the best way to prevent transmission of malaria (1,2).

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# Screening Blood Donors at Risk for Malaria: Reply to Hänscheid et al.

**To the Editor:** The letter to editor by Hänscheid et al. addresses our suggestion that polymerase chain reaction (PCR) could serve as a reference test for screening blood donations. At present, PCR is the most sensitive and specific method for parasite detection in malaria-endemic areas. However, additional measures should be taken into account, such as serologic testing, refining donor history, defining at-risk locations, and delimiting malaria-endemic areas. Therefore, we do not suggest that only PCR should be used as a reference method to exclude blood donors at risk, but it could help shorten the deferral period for blood donor (currently 3 years after an asymptomatic person leaves the malaria-endemic area). PCR should be accompanied by serologic tests and the elimination of actual or possible plasmodial infection in the blood donor.

In our laboratory, we use the indirect fluorescent antibody test (IFAT) for antigens of the four plasmodia species, in addition to PCR screening. At present, we have analyzed a total of 531 blood samples (406 more than the 125 described in our previous letter to the editor) from possible donors at risk for malaria, and only the five described in the letter were malaria positive. Moreover, 40% (50 of 125) of these sera were negative by IFAT (unpub. data), a fact that indicates the importance of having a complete donor history and being certain of the patient's origin in the context of malaria endemicity (i.e., several geographic areas without malaria transmission in some Central and South American countries could be excluded as malaria-risk areas; these areas coincide with sera negative by IFAT).

On the other hand, with a standard 450-mL blood donation, parasitemias <90 could test negative by PCR; but, as previously described, this technique should be accompanied by careful questioning, serologic testing, and eliminating parasites from the recipient during blood processing and storage.

We take for granted that, theoretically, any method would have to detect a single parasite per unit of blood to be safe and that little is known about the frequency of low parasitemias. In nature, and in accordance with the parasitologic definition of equilibrium between parasite and host (defined over thousands or millions of years according to different phylogenetic theories), one of the main strategies for parasite survival is sustained malaria transmission, which allows low parasitemias to be ingested by the anopheline vector (the amount of blood ingested by the female anopheline varies from 1.3 to 3.0 μL) (1). In this way, the amount of blood should have sufficient parasites to continue the cycle inside the vector. This fact explains the stability of malaria transmission during dry seasons. Plamodium falciparum infections can persist for at least 1 year in a substantial proportion (10%) of the host (2).

In two-thirds of the cases cited by Mungai et al. (3), the donor-screening process failed, illustrating the difficulties in obtaining accurate travel and immigration histories from donors. In this paper, serologic tests were positive retrospectively in 98% of tested donors, indicating that serologic tests should be a useful screening technique for malaria blood donors; 35% showed parasitemia in blood smears, a level that would have increased if the blood had been analyzed on the day of transfusion and not in retrospective study (after the degradation or deformation of the parasites or loss of staining of parasite chromatin).

Moreover, two of the three cases described by Slinger et al. (4) were in blood donors positive by microscopy or PCR (the other potential blood donor was not available for follow-up). These results show that parasites could have been detected in these cases, reflecting that all blood samples had detectable parasitemias.

Serologic tests in Spain indicate that approximately 50% of the referral donations could be used in transfusion, and travel histories should distinguish the specific destinations or the level of malaria transmission in the area. Most histories are based on the wide areas of transmission listed in travel guidelines. These data should decrease the cost of testing per blood donation when we add the value of the blood donation to the real cost of death prevented.

In conclusion, our initial results, now accompanied by serologic results, justify the exclusion criteria we first reported (5). Screening tests for blood donors (e.g., PCR) should be used as a reference technique that could shorten the deferral period for blood donors. In all well-reported cases (complete studies with follow-up) of transfusion-associated malaria described in Canada and the United States, PCR could have detected the parasites in blood.

Finally, the study of donors at risk could serve as indirect surveillance for asymptomatic infections and could play an important role in detecting autochthonous malaria transmission in the United States (5) or Spain where local anopheline vectors exist. An additional benefit for parasite detection is that it would permit the donor to be treated and locally acquired malaria to be eliminated. The Anopheline mosquito vectors of malaria still exist in the United States at levels sufficient to sustain malaria transmission, and dozens of cases of autochthonous malaria transmission have been reported in the United States over the past 15 years (6).

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## *Rickettsia aeschlimannii*: A New Pathogenic Spotted Fever Group Rickettsia, South Africa

**To the Editor:** Spotted fever group rickettsiae are increasingly recognized as agents of disease in residents of and tourists to South Africa (1). To date, two species, *Rickettsia conorii* and *R. africae*, which cause Mediterranean spotted fever (MSF) and African tick-bite fever (ATBF), respectively, have been associated with human disease in the region; ATBF is more frequently associated with travel (1). As different antibiotic regimens are recommended for the two syndromes, differentiating MSF from ATBF is important. Increasing evidence shows that the syndromes can usually be differentiated through clinical manifestations and epidemiologic characteristics (1).

We recently encountered a South African patient who, on returning from a hunting and fishing trip, discovered a *Rhipicephalus appendiculatus* tick attached to his right thigh and an eschar around the attachment site. The patient was aware of the risk of tick-transmitted disease; after removing the tick, immediately self-prescribed doxycycline. No further symptoms developed. However, as a precaution, the patient went to a local clinic, where a skin biopsy was taken from the eschar. This sample, together with the removed tick, was submitted to our laboratory. DNA extracts, prepared from an eschar biopsy and the tick, were incorporated into a polymerase chain reaction (PCR) assay specifically targeting a fragment of the rickettsial *omp*A (2). Sequence analysis of the amplification products showed both to be identical and to share >99% similarity with the *omp*A of *R. aeschlimannii*, a species not previously associated with human disease. Unfortunately, blood samples could not be collected at the time the patients first had symptoms; thus, investigation of a disseminated infection by PCR and serologic testing was not possible.

Although genotypically indistinguishable organisms had previously been detected in *Hyalomma marginatum* collected in Portugal and Zimbabwe, *R. aeschlimannii* was first characterized following its isolation from *H. marginatum* ticks in Morocco (3) and recently in Niger (4). This encounter was the first demonstration of its presence in South Africa and in *Rhipicephalus* ticks.

A lack of suitable clinical material prevented full evaluation of the pathogenic potential of *R. aeschlimannii* in this patient and prompt antibiotic intervention may have prevented evolution of the syndrome. Nonetheless, that *R. aeschlimannii* was transmitted to the patient and established a local infection leading to eschar formation provides clear, albeit preliminary, evidence of its virulence. Until further cases are encountered, allowing better characterization of the clinical manifestations associated with *R. aeschlimannii* infection and considering the agent capable of inducing either MSF or ATBF-like manifestations is crucial; neither of these syndromes can be associated with a specific causative agent without microbiologic identification. Our findings demonstrate that *Rickettsia* species first encountered in tick surveys are associated with human disease, and we should not assume that some *Rickettsia* species not have a pathogenic potential.

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## Age as a Risk Factor for Cutaneous Human Anthrax: Evidence from Haiti, 1973–1974

To the Editor: Few cases of anthrax have been reported in children, in part because most exposures to *Bacillus anthracis* occur in workplace settings. Questions about the susceptibility of children to *B. anthracis* infection were raised when cutaneous anthrax developed in a 7-month-

old child in New York City in 2001 after he was taken to visit his mother's workplace (1). No cases of anthrax were reported in persons <24 years of age in the 1979 inhalational anthrax outbreak in the Soviet city of Sverdlovsk, despite a presumed general population exposure (2). Such reports have led some investigators to postulate that young persons may be less susceptible to anthrax than older persons.

In 1974, the Center for Disease Control reviewed records on the occurrence of human anthrax in District Sanitaire des Cayes, Haiti, as part of an investigation of cutaneous anthrax in a Florida woman exposed to spore-contaminated goatskin drums she purchased in Haiti (3). In 1973, a total of 387 cases (7.6 per 10,000 population) were clinically diagnosed in District Sanitaire des Cayes; another 59 cases occurred in the first 4 months of 1974. All cases were the cutaneous form; gastrointestinal and inhalational anthrax are rarely, if ever, diagnosed in Haiti. The source of infection in these 446 patients could not be determined. Although cases of animal anthrax were rarely reported in Haiti because of a weak surveillance system, 96 (26%) of 368 Haitian goatskin handicraft items were found to be contaminated with *B. anthracis* during the 1974 investigation, suggesting that animal infections were not uncommon (4). Therefore, the source of the infection may have been meat and other products of value salvaged by local residents from anthrax-infected animals.

Age was reported for 366 of the 446 patients in District Sanitaire des Cayes (Table). The distribution of anthrax cases by age group was generally similar to that of the general population, except the proportion of cases in the 15- to 44-year age group was lower than the proportion of persons in that age group in the general population ($p<0.03$; chi square). In 124 patients for whom information was available, the cutaneous lesion was located on the head or neck (60 patients [48%]), the arm (31 [25%]), the trunk (23 [19%]), and the leg (12 [10%]); this anatomic distribution, reflecting the primary skin contact point of the organism, was similar in all age groups. However, determining whether the various age groups had differences in skin contact exposure leading to infection is difficult. The affected rural population lived in extreme poverty, typically in small huts with dirt floors and no safe water supply or latrine. Malnutrition was epidemic, and nothing edible was discarded. The crowded living conditions limited opportunities to maintain basic personal hygiene and made it likely that exposure to *B. anthracis*–contaminated materials was similar across all age groups. These previously unpublished age-specific anthrax attack rates from Haiti suggest that adults and children have similar susceptibility to cutaneous anthrax.

Table. Reported ages of persons with cutaneous anthrax, District Sanitaire des Cayes, Haiti, 1973–74

| Age group (yrs) | Anthrax patients No. (%) | General population No. (%) | Cases per 10,000 persons |
|---|---|---|---|
| <1 | 15 (4.1) | 16,361 (3.2) | 9.2 |
| 1–4 | 49 (13.4) | 59,291 (11.6) | 8.3 |
| 5–14 | 96 (26.2) | 120,532 (23.5) | 8.0 |
| 15–44 | 135 (36.9) | 235,164 (45.8) | 5.7 |
| 45–64 | 58 (15.8) | 64,882 (12.7) | 8.9 |
| ≥65 | 13 (3.6) | 16,669 (3.2) | 7.8 |
| All ages | 366 | 512,899 | 7.1 |

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Letters Editor: Patricia M. Quinlisk, Des Moines, Iowa, USA

# Candida and Candidiasis

**Richard A. Calderone, editor**

**American Society for Microbiology Press, Washington, 2001; 472 pages.**

Yeast of the genus *Candida* have exploded into prominence in recent years as opportunistic and nosocomial fungal pathogens. However, the most recent textbook on these organisms was written in 1988. Candida and Candidiasis is a worthy successor in providing comprehensive information on the biology of these organisms.

A total of 28 chapters cover the general properties, virulence factors, cell biology, immunity, genomics, diseases, and laboratory aspects of *Candida* species, with particular emphasis on its most prominent member, *Candida albicans*. The strongest chapters are those covering research aspects of these organisms. Complex subjects like the chemistry of the cell wall, host recognition and adherence, the cell biology of the yeast-hyphal transformation, and extracellular hydrolases as virulence factors in *C. albicans* are well summarized with clear, useful graphics and current references. The book is beautifully laid out, with a series of color plates that help describe phenotype switch variants and chromosome maps.

The clinical chapters appear rather superficial for an infectious diseases clinician but may be useful to a student seeking basic material. The chapter on identification and subtyping contains information available in other sources for less than the cost of this book. A discussion of current practices in antifungal susceptibility testing of *Candida* species would have been helpful. Chapters 2 and 4 contain repetitious material, including photographs of *C. dubliniensis*. A consolidated chapter on the epidemiology of *Candida* infections should be considered for the next edition. The chapters covering the cell biology are most useful, either as a comprehensive overview or as a reference text for researchers and students interested in the biology of these organisms.

**Mary E. Brandt**

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# Food Safety for Professionals (Second Edition)

**Mildred M. Cody and M. Elizabeth Kunkel**

**American Dietetic Association Chicago, Illinois; 198 pages**

Drs. Cody and Kunkel have compiled an informative overview of food safety issues that is targeted toward dietetics professionals in particular but is also useful for food safety professionals. The guide contains many of the standard elements found in dietetics textbooks, including charts of infectious agents, information on specific foods and safety concerns, and basic food safety programs. The authors have wisely amended the standard textbook approach by including information on consumer needs and behaviors, a review of food safety surveillance programs, and a discussion of food safety laws and regulations. This edition includes additional chapters on suggestions for continuing education for dietetics professionals and an expanded list of resources, including online references.

This guide includes many useful details in a understandable format. The text is replete with tables (e.g., Descriptions of Specific Foodborne Bacterial Pathogen; Major Food Laws in the United States), which make the wealth of information easily readable. An extensive glossary specific to dietetic practice is included. The text also contains a continuing education self-assessment instrument for dietitians.

The breadth of the text is both its strength and weakness: a vast amount of material is covered, but inevitably, general statements are bound to leave out subtleties useful to the reader. In addition, foodborne illnesses caused by bacterial pathogens are emphasized; therefore, much of the discussion is focused on control measures for bacteria. The text incorrectly states, "…bacteria cause most of the cases of foodborne illness in the United States…." Most cases of foodborne illnesses are caused by unidentified agents. Of the illnesses of known origin, most are caused by viruses (1). On the other hand, the authors wisely include a discussion of parasites, an often overlooked as a cause of foodborne disease.

Similarly, the statement "FDA can order a product recall (or seize goods in the field)" on page 104 is inaccurate. While FDA can seize goods or request that a firm initiate recalls of food products, the agency's authority does not currently extend to mandatory recalls for most foods. FDA can, however, require a recall of infant formula under certain circumstances. The text would benefit from a deeper discussion of the role of the respective federal agencies in protecting the U.S. food supply. In addition, several important issues are not addressed or are not discussed thoroughly (e.g., global food safety considerations, the national food safety system, HACCP regulations for meat and poultry, seafood, and juice).

To their credit, the authors include a discussion of chronic sequelae of foodborne infections, an important area of consideration, particularly for dietitians.

The text makes heavy use of Internet references. These references provide an abundance of current information but risk becoming out-of-date if the sites disappear or are not routinely updated.

Overall, the book is a valuable resource for its soup-to-nuts information approach. The book, a gold mine

of useful information for dietitians, provides good one-stop-shopping for infectious disease scientists and professionals wishing to learn about the world of food safety.

**Camille Brewer***
**and Arthur P. Liang†**

Food and Drug Administration, College Park, MD; and †Centers for Disease Control and Prevention, Atlanta, GA

### Reference

1. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-Related illness and death in the United States. Emerg Infect Dis 1999:5;607–25.

### Correction, Vol. 8, No. 7

In Time-Space Clustering of Human Brucellosis, California, 1973–1992, by G. Fosgate et al., an error appears in the results section. The corrected sentence appears below and online at http://www.cdc.gov/ncidod/EID/vol8no7/01-0351.htm .

The *Brucella* species was identified in 229 (55%) of the 416 cases analyzed. *B abortus* was isolated from 39 cases, *B. melitensis* from 181, and *B. suis* from 9.

We regret any confusion this error may have caused

### Emerging Infectious Diseases Policy on Corrections

The Emerging Infectious Diseases journal wishes error-free articles. To that end, we

1) Make corrections as quickly as we become aware of errors

2) Publish corrections online and in print. Online, we correct the error in the article it occurred with a note that the article was corrected and the date of correction. In print, we prominently publish a full correction, printing all needed information, and provide the URL of the corrected online article for reprints.

For additional information on corrections, send e-mail to eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

# JOURNAL BACKGROUND AND GOALS

### What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

### Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

### What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

## About the Cover

**Eugène Delacroix (1798-1863).**
**"Arab Horses Fighting in a Stable." 1860.**

**Oil on canvas. Photo: Gerard Blot.**
**Copyright Réunion des Musées Nationaux/Art Resource, NY**

**Louvre, Paris, France**

From his early years, Delacroix, like his contemporary Théodore Gericault, was attracted to the savagery of wild animals. In a note written in Morocco, Delacroix mentions a scene of fighting horses. Among the precedents for this kind of wild-animal imagery was the antique group "Lion Attacking a Horse" (Rome, Mus. Conserv.), which was said to have been particularly admired by Michelangelo and which was copied in stone by Peter Scheemakers (1740; Rousham Park, Oxon). George Stubbs used the wild-animal theme within naturalistic settings in several paintings, for example, in "Horse Attacked by a Lion," (1770; London, Tate), of which Gericault made at least one copy (1820/21; Paris, Louvre).

In "Arab Horses Fighting in a Stable," his version of the subject, Delacroix was able to synthesize the classical with the exotic, with his studies of ecorché (French for flayed bodies), with the example of English art, and with the work of Rubens—Delacroix owned Pieter Claesz Soutman's engravings of Rubens' paintings of hunts. In 1847, Delacroix described two of these engravings in detail, indicating how highly he valued the elements of movement, variety, and unity. Of Delacroix' three great lion hunts, three have survived, a fragment (1855, now in Bordeaux, France) and two complete paintings: one from 1858 (now in Boston, Massachusetts) and one from 1861 (now in Chicago, Illinois); the latter is the most spacious and free in its handling of circular, dancelike movements that suggest a perpetual struggle—one of the underlying themes in which form and content are inseparable.

From The Dictionary of Art, Macmillan, NY, NY, 1996.

## Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at http://www.cdc.gov/eid.

## 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 e-mail 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. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

**Biographical Sketch**. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

**References**. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

**Tables and Figures**. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

**Manuscript Submission.** Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Submit an electronic copy (by e-mail) to the Editor, eideditor@cdc.gov.

## 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) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Synopses**. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Research Studies**. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

**Policy and Historical Reviews**. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a 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.

**Commentary**. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

**Another Dimension**. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

**Letters**. This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**Book Reviews**. Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

**News and Notes**. 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.) In this section, we also include summaries (500–1,000 words) of emerging infectious disease conferences. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.