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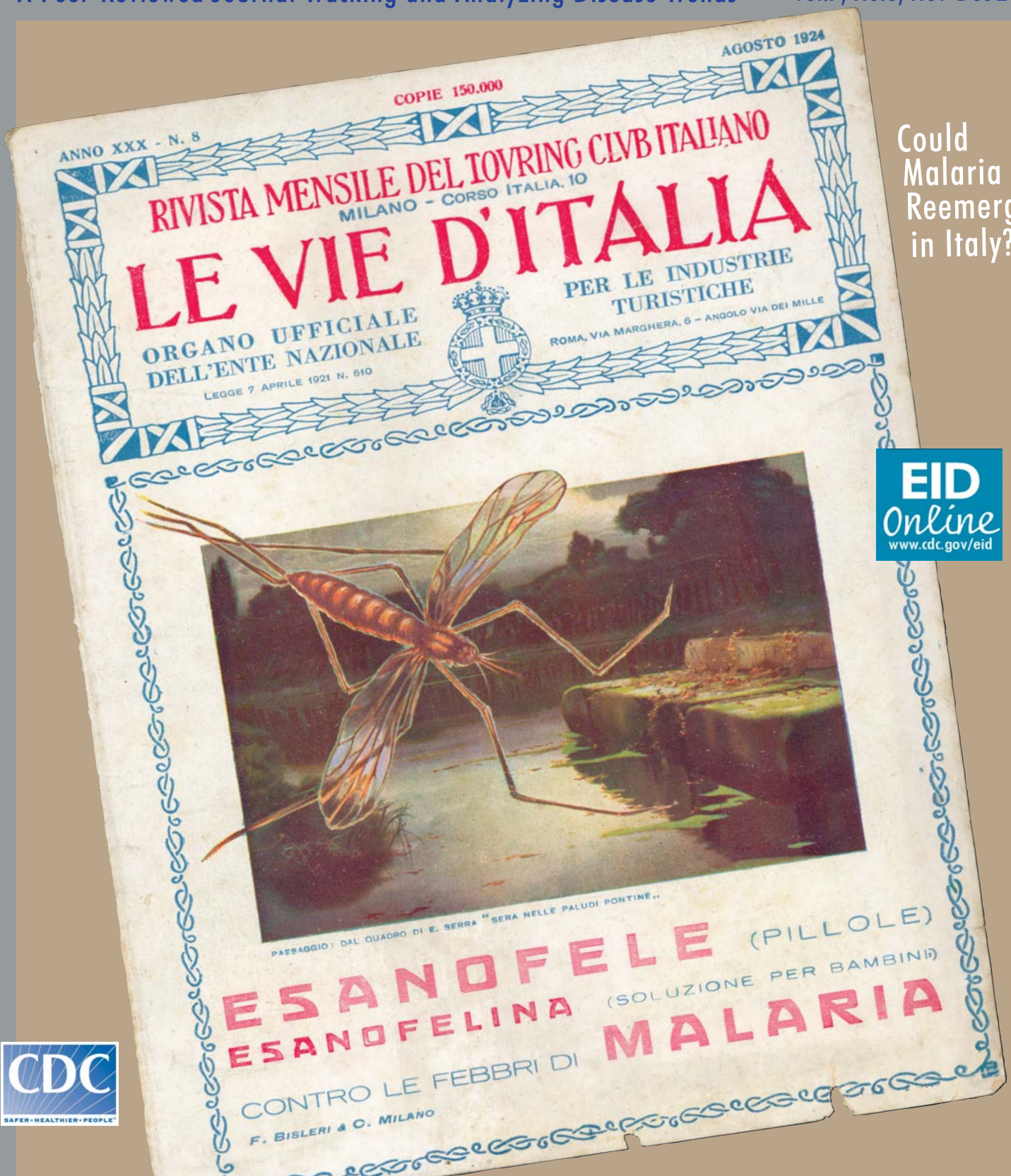


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Cover of Le Vie d'Italia magazine from 1924, provided courtesy of Dr. Guido Sabatinelli.

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## News and Notes

# Could Malaria Reappear in Italy?

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Because of concern about the possible reintroduction of malaria transmission in Italy, we analyzed the epidemiologic factors involved and determined the country's malariogenic potential. Some rural areas in central and southern Italy have high receptivity because of the presence of potential malaria vectors. *Anopheles labranchiae* is probably susceptible to infection with *Plasmodium vivax* strains, but less likely to be susceptible to infection with *P. falciparum*. Its vulnerability is low because of the low presence of gametocyte carriers (imported cases) during the season climatically favorable to transmission. The overall malariogenic potential of Italy appears to be low, and reintroduction of malaria is unlikely in most of the country. However, our investigations showed that the malaria situation merits ongoing epidemiologic surveillance.

At the end of World War II, malaria was still present in vast areas of Italy, mainly in the central and southern regions and major islands and along northeastern coastal areas, with offshoots of hypoendemicity in the Pianura Padana (1). The three vectors were *Anopheles labranchiae* Falleroni and *An. sacharovi* Favre, both belonging to the so-called *maculipennis* complex, and *An. superpictus* Grassi (2). *An.labranchiae* was the principal vector in the central and southern coastal areas, Sicily, and Sardinia. In the two islands, the species was found as high as 1,000 meters above sea level. *An. sacharovi* was present along much of the coastal area and in Sardinia, but was most important as vector in the plains of the northeastern Adriatic coast, where *An. labranchiae* was absent. *An. superpictus* was considered a secondary vector in central and southern Italy and Sicily. In some interior areas of the Pianura Padana, where none of the three vectors was present, low levels of endemicity were probably maintained by other species belonging to the *maculipennis* complex.

A malaria eradication campaign launched in 1947 led to interruption of transmission of *Plasmodium falciparum* malaria throughout Italy within 1 year (3). Indoor treatment with DDT (2 g of active ingredient per $m^2$) of houses, stables, shelters, and all other rural structures continued into the mid-1950s and even later in some hyperendemic areas. In Sardinia, where transmission was particularly high, a special program was carried out to eradicate the vector (4). The last endemic focus of *P. vivax* was reported in the province of Palermo, Sicily, in 1956 (5), followed by sporadic cases in the same province in 1962 (6). The World Health Organization declared Italy free from malaria on November 17, 1970. Since then, almost all reported cases have been imported, but their number has risen steadily over the last decade(7,8).

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In 1997, a case of introduced malaria occurred in a rural area of Grosseto Province, the first since the eradication of malaria from Italy (9). This event, along with the occasional presence of *Plasmodium* carriers who contracted the disease in malaria-endemic areas and the increasing number of immigrants from malaria-endemic countries entering Italy, raises concern about the possible reappearance of malaria foci in certain areas. We evaluate the malariogenic potential of Italy and assess the risk for malaria transmission in some areas, decades after the last analysis of the problem (10,11).

## Material and Methods

The risk of malaria being reintroduced to an area can be calculated by determining its "malariogenic potential," which is influenced by three factors: receptivity, infectivity, and vulnerability. Receptivity takes into account the presence, density, and biologic characteristics of the vectors; infectivity is the degree of susceptibility of mosquitoes to different *Plasmodium* species; and vulnerability is the number of gametocyte carriers present in the area.

### Receptivity

To evaluate Italy's receptivity, we analyzed historical data and the results of entomologic surveys carried out in Italy as part of epidemiologic investigations over the last 20 years. The vectorial capacity of some Italian populations of *An. labranchiae* was also estimated by the MacDonald formula (12).

### Infectivity

The possibility that the sporogonic cycle of the various *Plasmodium* species may be completed within a vector is defined as infectivity. Only a few species of the *Anopheles* genus are capable of becoming infected and transmitting malaria. Furthermore, for genetic reasons, even mosquito populations of the same species can differ in sensitivity to plasmodia (13) or may be completely resistant to infection with plasmodia from the same species but different

geographic areas. Infectivity in a mosquito population is a determining factor in the assessment of malariogenic potential in a given area. We analyzed data in published studies to evaluate the infectivity of Italian vectors.

### Vulnerability

Vulnerability in a given territory is determined by the number of gametocyte carriers during the period in which malaria transmission is possible. To determine the degree of Italy's vulnerability, a sample of malaria cases reported from 1989 to 1996 was selected on the basis of spatial and temporal risk factors for the transmission of malaria. Malaria cases reported in Italy in 1997 were also analyzed. Because of the limited distribution of vectors potentially capable of transmitting malaria, we considered only cases in Tuscany, Campania, Abruzzo, Molise, Basilicata, Apulia, Calabria, Sicily, and Sardinia. In the past, the season of malaria transmission in central and southern Italy lasted from June to late September for *P. vivax* and from July to early September for *P. falciparum*. We therefore selected cases reported from June through September.

## Results

### Receptivity

After their drastic reduction as a result of the DDT campaign, the endophylic anopheline species have begun to reproduce again and in many cases have reached preintervention densities (14). Of the anopheline species that had been vectors of malaria in Italy, only *An. labranchiae* and *An. superpictus* are still present in epidemiologically relevant densities (14). In other European and Mediterranean countries, other anophelines have been considered secondary (*An. atroparvus* and *An. melanoon*) and occasional (*An.algeriensis*, *An. hyrcanus*, and *An. claviger*) malaria vectors. *An. sergenti*, a north African species, was implicated in the 1960s in the transmission of a few sporadic case of malaria on the island of Pantelleria (15).

### Distribution and Density of Potential Vectors

In northern Italy, in particular the northwestern regions (Veneto and Emilia) where *An. sacharovi* was present, the last specimens of the vector were found in the province of Rovigo (16); in the last 30 years there have been no further records. No *An. sacharovi* larvae or adults were recorded in a recent survey along the northwestern coast of Italy (17). However, areas with epidemiologically relevant anopheline densities still exist in Tuscany (only in Grosseto Province), Calabria, Puglia, Sicily, and Sardinia (14), where hydrogeologic or environmental characteristics are conducive to the development of vectors (Table 1). Residual populations of *An. labranchiae* and *An. superpictus* could still be present along the coasts of Abruzzo, Molise (east coast), Campania, and Basilicata (west coast), but no relevant densities have recently been reported.

### Vectorial Capacity (VC)

The high density of anopheline populations reported in some areas of Italy does not necessarily imply the resumption of malaria. Other entomologic factors must be taken into consideration to estimate the risk of transmission. The VC of a mosquito population is the measure used in epidemiology to estimate risk in various geographic areas. It expresses the number of potentially infective bites that originate daily from a case of malaria in a given area or, more precisely, from a carrier of gametocytes capable of infecting all the

Table 1. Distribution and density of *Anopheles labranchiae* and *An. superpictus* in five regions of central, southern, and insular Italy

| Region | At-risk areas | Vector | Larval breeding sites[a] | Vector density and capacity[b] |
|---|---|---|---|---|
| Tuscany | Grosseto province: areas of intensive rice cultivation (S. Carlo, Principina and S. Donato, Orbetello) | *An.labranchiae* | Rice fields, agricultural and land reclamation canals, wells. Larval densities in rice fields 5-10 larvae/sample, elsewhere 0.5-1 larvae/sample | 100-1,000 per animal shelter; 180-200/person /night. VC in rice fields: *P. falc.* 7-26; *P. vivax* 8.3-32.5; VC in natural breeding sites: *P. falc.* 0.8-2.9; *P. vivax* 0.96-3.3 |
| Apulia | Coastal plains of the Adriatic side, from Lesina Lake to Candelaro River | *An.labranchiae* | Land reclamation canals, pools for agricultural purposes. Larval densities 0.02-0.05 larvae/sample | 20-30 per animal shelter |
| Calabria | Coastal plains of the Tirrenian and Ionian sides and the close hinterland | *An. labranchiae* *An. superpictus* | Larval densities: *An. labranchiae* 0.5-1 larvae/sample *An. superpictus* 0.06-0.1 larvae/sample | 20-500 *An. labranchiae*, 2-10 *An. superpictus* per animal shelter. 10-20 *An.labr.*/person/ night. VC of *An. labranchiae* for *P. falciparum* 0.8-8.9 |
| Sicily | Rural coastal and hilly areas of the whole region | *An. labranchiae* *An. superpictus* | Rivers, streams, pools, and canals for agricultural purposes. Larval densities of *An. labranchiae* 0.03 to 0.5 larvae/sample | 10-200 *An. labranchiae* per animal shelter |
| Sardinia | Rural coastal and hilly areas of the whole region | *An. labranchiae* | Mainly rivers and streams; ponds, artificial pools, rice fields and irrigation canals. Larval densities 1 to 10 larvae/sample | 5-40 per animal shelter |

[a] Figures refer to areas considered as "at risk" for malaria reintroduction during surveys carried out from 1994 to 1996.
[b] Calculated at a mean temperature of 25°C (July to August), assuming a sporogonic cycle of 11 days for *P. falciparum* and 10 days for *P. vivax*.
VC = vectorial capacity.

receptive mosquitoes that feed on the carrier. VC is influenced by three factors: the anthropophily, longevity, and density of the vector. The few recent estimates of the VC of Italian anopheline populations have been limited to *An.labranchiae* (14,18). The first attempt was made in 1978 in Calabria: VC was reported as 0.82 to 8.9, with an average density of 16 bites per person per night (Coluzzi A. and M., unpub. data). In 1994, in a large area of rice cultivation in Tuscany (Grosseto Province), VC was very low in early July, constituting no real risk for malaria transmission (<0.01 for both *P. falciparum* and *P. vivax*). At the beginning and especially the end of August, VC was high (8 to 32.5), especially for *P. vivax*, which has a shorter sporogonic cycle than *P. falciparum* (VC 7 to 26). This high VC is undoubtedly influenced by the high number of bites per person per night (>200) reported in the area (14). In 1998 in the same province but in areas where only natural anopheline breeding sites are reported, we calculated the following VCs from mid-July through the end of August: *P. falciparum* 0.8 to 2.9 and *P. vivax* 0.96 to 3.3 (<10 bites/person/night) (18).

### Infectivity

As *Plasmodium* species have long been eradicated in Italy, it is essential to determine whether local vectors are still sensitive to infection with *Plasmodium* from other areas where malaria is present. Few tests of infectivity have been carried out with potential Italian vectors. There are numerous difficulties in rearing mosquitoes of the *An. maculipennis* complex, obtaining blood with vital gametocytes, and setting up an efficient artificial system that anophelines bite. To resolve these technical difficulties, some samples of *An. atroparvus* and *An. labranchiae* were captured in Italy in the 1970s and transported on several occasions to Kenya, where they were induced to bite *P. falciparum* carriers (19,20). In none of the mosquitoes did the plasmodia carry out the entire cycle and reach the salivary glands. These susceptibility tests were, of course, carried out with an extremely low number of samples and are insufficient to confirm that Italian anopheline populations are entirely resistant to infection with African strains of *P. falciparum*. Nevertheless, data on the resistance of Italian anopheline populations to tropical African *P.falciparum* strains agree with other observations made in England (21) and Portugal (20) on local *An. atroparvus* populations.

Populations of *An. atroparvus* from the eastern Russian Federation were sensitive to *P. vivax* strains from Southeast Asia (22), and populations from Romania were successfully infected with *P. vivax* strains from Korea (23). The marked tendency of Italian populations of *An. atroparvus* to bite animals, together with susceptibility assays carried out so far (19), does not indicate that this species is a malaria vector in Italy. As for *An. labranchiae*, this particularly anthropophilic Mediterranean species can certainly transmit *P. vivax,* as shown by the 1971 epidemic in Corsica (24), the cases reported in Greece during 1975–76 (25), and the recent sporadic case in Grosseto Province (9). The susceptibility of *An.superpictus* to *P. falciparum* of African origin has not been tested, but this mosquito is probably sensitive, as it belongs to the subgenus *Cellia*, to which the principal African malaria vectors also belong.

### Vulnerability

Of 885 cases reported in 1997, only 88 (9.9% of the total) were reported from the nine regions at risk (Table 2). A total of 25 cases (2.8%) occurred during the season favorable to malaria transmission: 15 from *P. falciparum*, 9 from *P.vivax*, and 1 from *P. malariae*. Most of the patients (64%, n=16) lived in Tuscany. Considering that the highest anopheline mosquito densities were reported in this region, these results are cause for concern. On the other hand, the samples were quite small (16 patients) and other factors need to be considered. First is the number of patients who live in rural areas, since these are the only areas where the vector can come into contact with a gametocyte carrier. The analysis of samples shows that most of the 25 at-risk patients (72%, n=18) lived in an urban area. Another factor is the length of exposure of the malaria patients to mosquito bites during the disease or the length of their stay in a malaria-endemic area. In fact, all the patients received hospital care in urban areas, which would certainly limit mosquito-human contact. However, the factor that most affects a territory's vulnerability is the number of gametocyte carriers—the only persons who can infect mosquitoes—and the length of their potential exposure to mosquitoes. Of patients who lived in areas at risk and had contracted malaria during a period theoretically favorable to transmission, only eight became gametocyte carriers (six of them carriers of *P. vivax*). These carriers represent 0.7% of all malaria cases reported in Italy in 1997 and 4% of all the gametocyte carriers. In these patients, the average time between appearance of symptoms and malaria diagnosis (when therapy began) was 8.2 days, which is the period when patients could have been a source of infection for mosquitoes. The cases reported from 1989 to 1996 show similar results: of 5,012 cases, 522 (10.4%) occurred in central and southern Italy; only 184 of these occurred during high-risk months (June–September). Of 30 gametocyte carriers, 27 were of *P. vivax*, 2 of *P. falciparum*, and 1 of *P. ovale.*

## Conclusion

We investigated Italy's malariogenic potential and the possibility of a recurrence of transmission there. Our results indicate the following conclusions. First, some rural areas in central and southern Italy have high receptivity because of the presence of potential malaria vectors with VC. The figures for VC were obtained by collecting mosquitoes on persons exposed to mosquito bites without any protection (14). These data are purely theoretical, as it would be quite

Table 2. Italy's vulnerability to malaria during the season favorable to malaria transmission (June to September) from 1989 to 1997

| Year | Total no. of malaria cases | No. of cases in at-risk regions[a] | No. of cases in at-risk regions and favorable season | Total no. of gametocyte carriers | No. of gametocyte carriers in at-risk areas and season |
|---|---|---|---|---|---|
| 1989-1996 | 5012 | 522 | 184 | 646 | 30 |
| 1997 | 885 | 88 | 25 | 148 | 8 |

[a] Nine regions in central, southern, and insular Italy.

unlikely for a person to remain exposed to mosquito bites for long without taking preventive steps. For comparison with the VC calculated in Italy with that of malaria-endemic areas, the VC of *P. falciparum* reaches values >10 and in some cases >30 in the hyperendemic conditions in many areas of the African savanna.

However, even a level of 0.1 (the average production of an infective bite from a malaria patient every 10 days) appears sufficient to maintain hyperendemicity, once the number of carriers of *P. falciparum* gametocytes reaches 50% of the population. The critical VC value (i.e., the level below which malaria does not remain endemic) has been calculated for the Garki region (Nigeria, State of Kano) as 0.022, or an average production of about 1 infective bite from a malaria patient every 50 days (26). In theory, therefore, the VC in some areas of Italy is epidemiologically significant, and these areas could become receptive.

Second, *An. labranchiae* is susceptible to infection with *P. vivax* strains from malaria-endemic areas, while infection with tropical African strains of *P. falciparum* seems less likely. Third, Italy's vulnerability is low because of the low presence of gametocyte carriers during the season climatically favorable to transmission in areas at risk. These figures are certainly underestimates, however, as in some regions of central and southern Italy not all malaria cases are reported, and the number of migrants from countries with endemic malaria, who come to Italy to work, is constantly increasing (7,8).

The overall malariogenic potential of Italy appears to be low, and malaria reintroduction is unlikely in most of the country. Sporadic autochthonous *P. vivax* malaria cases may occur but only in limited rural areas, where high densities of *An. labranchiae* have been reported. These results indicate the need for more epidemiologic surveillance, especially as the Italian situation is extremely dynamic and changeable. Sociopolitical factors, in particular, could lead to substantial changes in the flow of immigrants from endemic malaria areas, and environmental factors could result in changes in the density and distribution of vector populations.

Furthermore, the continuous contact of strains of exotic plasmodia with potential mosquito vectors could lead to long-term selection or adaptation of strains capable of developing in Italian mosquitoes. The possible presence in rural central and southern Italy of potential *P. vivax* carriers (e.g., immigrants from Asia and Africa hired as seasonal workers) is of concern.

A possible episode of autochthonous malaria transmission in Italy would not have serious health consequences, as it could easily and quickly be controlled by the National Health Service. The impact on Italy's image, however, could be serious at the international level. From an economic standpoint, reports of malaria cases would undoubtedly affect Italy's tourist industry.

To prevent and manage indigenous malaria cases in areas where the density of the vector is substantial, regional or local centers should be established with experts competent in epidemiologic surveillance and malaria control. These centers should also monitor the movements of malaria *Plasmodium* carriers in the country and assess the risk for malaria transmission in different regions.

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# Developing New Smallpox Vaccines

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New stockpiles of smallpox vaccine are required as a contingency for protecting civilian and military personnel against deliberate dissemination of smallpox virus by terrorists or unfriendly governments. The smallpox vaccine in the current stockpile consists of a live animal poxvirus (*Vaccinia virus* [VACV]) that was grown on the skin of calves. Because of potential issues with controlling this earlier manufacturing process, which included scraping VACV lesions from calfskin, new vaccines are being developed and manufactured by using viral propagation on well-characterized cell substrates. We describe, from a regulatory perspective, the various strains of VACV, the adverse events associated with calf lymph-propagated smallpox vaccine, the issues regarding selection and use of cell substrates for vaccine production, and the issues involved in demonstrating evidence of safety and efficacy.

An attack on the United States with a biological or chemical weapon is an eventuality that must be actively addressed (1,2). Civilian and military public health agencies are preparing to cope with biological terrorism and warfare through efforts to stockpile vaccines and antibiotics needed to protect against diseases caused by potential biological weapons. New vaccines, such as a new smallpox vaccine, are being actively developed, and other biologics are needed to counteract the effects of these weapons on the domestic population and military forces.

Smallpox (*Variola major*) is a particularly dangerous biological weapon threat because of its clinical and epidemiologic properties (2,3). This virus can be manufactured in large quantities, stored for an extended period of time, and delivered as an infectious aerosol. Evidence indicating that potential enemies of the United States possess smallpox virus has led to concerns about the susceptibility of U.S. troops and civilians to the virus and the need to develop defense strategies (4). With the success of the World Health Organization (WHO) campaign in the 1970s to eradicate naturally occurring smallpox and the subsequent discontinuation of vaccination, a large proportion of the population has no immunity. Case-fatality rates could be higher than 25% if smallpox were released as a bioterrorist weapon (5).

The U.S. stockpile of smallpox vaccine is maintained by the Centers for Disease Control and Prevention (CDC). These decades-old doses may be inadequate to meet the vaccination needs projected after a bioweapon incident. The number of doses needed in various scenarios has recently been discussed (6). In 1972, for example, a single case of smallpox in Yugoslavia required 18 million doses of vaccine to stop the spread of disease (7). At least 40 million doses of vaccine are projected to be needed to respond effectively to a terrorist attack in the United States (6).

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## Smallpox Vaccine

The only commercially approved smallpox vaccine available for limited use in the United States is Wyeth Dryvax. This vaccine is a lyophilized preparation of live *Vaccinia virus* (VACV), made by using strain New York City calf lymph (NYC_CL), derived from a seed virus of the New York City Board of Health (NYCBH) strain of VACV that underwent 22 to 28 heifer passages. The vaccine consists of lyophilized calf lymph containing VACV prepared from live calves. The animals were infected by scarification, and the skin containing viral lesions was physically removed by scraping. The lyophilized calf lymph type vaccine is reconstituted with a diluent containing 50% glycerin, 0.25% phenol, and 0.005% brilliant green. Vaccine prepared by this traditional manufacturing technique of harvesting VACV from the skin of cows (and sheep) was used in most regions of the world during the smallpox eradication campaign. The facilities, expertise, and infrastructure required for producing the virus in this way are no longer available. Wyeth Laboratories discontinued distribution of smallpox vaccine to civilians in 1983(8).

This live-virus vaccine also caused rare but serious adverse reactions and common local reactions. Effective vaccination, classified as a "take," was indicated by the observation of a pustular lesion 6 to 10 days after vaccination at the injection site. This lesion represented a localized infection and was associated with the generation of an immune response. The vaccine take rate has been generally accepted as a correlate of vaccine efficacy. Specifically, there is a direct relationship between the intensity and extent of virus multiplication and the magnitude and duration of antibody response (9-13). The vaccine take rate of lesion formation for the currently stockpiled vaccine is >90%. Intradermal and intramuscular administration of VACV vaccine produces less severe local reactions, thus decreasing the risk of inadvertent autoinoculation or transmission; however, this inoculation route produces substantially lower responses as measured by enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody testing (9,13).

### Adverse Events Associated with VACV Vaccine

The complications arising from smallpox vaccination were well documented during the vaccination program (14-20). Dermatologic and central nervous system disorders were the most frequently recognized adverse events. Dermal complications included vaccinia necrosum, a complication with case-fatality rates of 75% to 100% that occurred almost exclusively in persons with cellular immunodeficiency (21). Eczema vaccinatum was associated with case-fatality rates of up to 10% overall and 30% to 40% in children <2 years of age (22). Generalized vaccinia was reported and probably resulted from rare bloodborne dissemination of virus in normal persons. Erythematous urticarial eruptions occurred in 1% of all primary vaccinees, and rarely, Stevens-Johnson syndrome occurred. Rarer diseases such as pericarditis (23), arthritis (24), and malignant tumors at vaccination scars (25) have been described in case reports.

During the U.S. smallpox vaccination program, approximately seven to nine deaths per year were attributed to vaccination, with the highest risk for death in infants. Most of these infant deaths were attributed to postvaccinal encephalitis (26). Most primary vaccinations in the United States were administered to children, so less is known about adverse events in adults. Rates tended to be higher in primary vaccinees and also in certain countries such as Austria and Denmark, where strains were used that may have been more virulent.

Of important concern is inadvertent administration of vaccine to persons who are immunodeficient or have other underlying contraindications to VACV vaccination, such as pregnancy. Administration of vaccine in the context of mass vaccination for outbreak control increases the risk for serious adverse events, since careful screening for vaccine contraindications would be problematic. Cultures of the vaccination sites of primary vaccinees have yielded positive cultures from days 3 through 14 after vaccination. Thus, transmission of VACV to close contacts of vaccine recipients does occur (27-30) and, in light of the global HIV epidemic and the large prevalence of patients on immunosuppressive therapy, constitutes a serious concern (31).

### Development of New VACV Vaccines

During the early eradication campaign, a number of studies were undertaken to determine the factors that rendered the smallpox vaccine potent and stable. WHO and its Expert Committee provided the initial recommendations for smallpox vaccines in 1959 and updated them in 1965, defining testing procedures and standards, including a required pock count of $1 \times 10^8$ PFU/mL of undiluted vaccine (32). In 1967, the National Institutes of Health published more stringent requirements, including the use of a national reference vaccine preparation.

#### Selection of Strain

The smallpox eradication campaign used vaccines derived from many VACV strains. In the United States, these included the New York City calf lymph (NYC_CL) and New York City chorioallantoic membrane (NYC_CAM) strains, both of which were derived from a seed virus of the NYCBH strain. Strains derived from the NYCBH strain caused lower rates of adverse events, especially encephalitis. Other strains used frequently in the global eradication program were EM-63 (USSR) and Temple of Heaven (China). The Lister or Elestree (United Kingdom) strain, prepared on the skin of sheep, was used extensively in Europe and other parts of the world. The Lister strain, which appeared to cause less illness than some of the other vaccine strains, was distributed by the WHO International Reference Centre to production laboratories for use as seed lots. By 1968, 71 producers used 15 principal strains of VACV, in addition to some unknown strains. From 1968 to 1971, the Lister strain became the most widely used throughout the world (33).

The exact origin and lineage of many of these strains and their relation to each other are not clear; however, all these strains (that were used in settings where smallpox actually occurred) were effective in eliminating the disease. With regard to efficacy, this observation suggests a degree of latitude in the selection of a VACV strain to provide protective immunity. From a scientific and regulatory perspective, many strains of VACV should be appropriate for a new vaccine as long as the manufacturer can demonstrate that the new vaccine is safe and elicits a "take" and an immune response analogous to that observed for the present licensed vaccine.

#### Selection of Cell Substrate

Historically, most manufacturers produced smallpox vaccine in live animals. However, this harvesting method has important limitations: it is prone to contamination with bacteria and other adventitious agents, and the antigenic and allergenic character of the accompanying animal protein can potentially result in sensitization and allergic reactions. Thus, the use of a well-characterized cell substrate for vaccine production has some potential advantages. The *Orthopoxviridae*, including *Vaccinia*, generally replicate on a wide range of candidate vertebrate fibroblast cell lines. The choice of a well-characterized or easily tested cell substrate for vaccine production can help expedite the review process (34). Issues associated with cell substrates that have been used for the manufacture of licensed live-virus vaccines may be the easiest to anticipate and address.

Use of primary cell substrates, particularly embryonated chicken egg-produced smallpox vaccine, would help to address major issues with regard to preparation of vaccine in cell culture. Cells derived from embryonated chicken eggs (especially chicken embryo fibroblasts) have been used in preparing many safe biological products, including vaccines. A variety of methods to ensure product safety can be evaluated rapidly with a high degree of confidence.

Candidate cell substrates for a new smallpox vaccine also include continuous cell lines or diploid cell strains of human or animal origin. For human cell substrates, the source of cells should be clearly described, including the tissue or organ of origin, ethnic and geographic origin, age, gender, and general physiologic condition, as well as the health or medical condition of the donor, if known. For animal cell substrates, description of the source should include species, strains, breeding conditions, tissue or organ of origin, geographic origin, age, gender, and general condition of the original donor.

The Food and Drug Administration (FDA) has licensed live-virus vaccines, such as varicella and rubella, prepared in diploid cell substrates (e.g., MRC-5, WI-38). Recently,

MRC-5 was used as a cell substrate for the preparation of an experimental smallpox vaccine under a Phase 1 trial (9). Another diploid cell strain, FrhL-2, has been used as a cell substrate for rotavirus vaccine and other live-virus vaccines tested in human clinical trials. The FDA experience in evaluating live-virus vaccines prepared in these diploid cell substrates makes the selection and use of such cell substrates potentially suitable for manufacture of a smallpox vaccine.

The continuous cell line Vero has been used to prepare a U.S.-licensed inactivated virus vaccine, the inactivated polio vaccine. Although the FDA has not yet licensed a live-virus vaccine manufactured in a continuous cell line, international experience with Vero cells suggests that they may be a suitable substrate for a smallpox vaccine. Issues pertaining to Vero cells as a substrate for live vaccines, including tumorigenic potential, were discussed at the May 2000 Vaccines and Related Biological Products Advisory Committee meeting (35). Further information regarding cell substrates can be obtained in the FDA document "Draft Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993). An FDA letter to vaccine manufacturers concerning the use of Vero cells was recently issued (http://www.fda.gov/cber/letters.htm).

Any new smallpox vaccine ideally should not be less immunogenic than animal-derived vaccines. The properties of *Poxviridae* host range, virulence, and genome composition have been shown to change upon many passages in tissue culture cells. To retain the properties that make VACV a good vaccine against smallpox, the number of passages in the cell culture substrate should be kept to a minimum.

LC16m8, an attenuated VACV strain developed by Japan in 1975 for primary vaccination, was derived by passing the Lister strain 36 times through primary rabbit kidney cells at low temperature (30°C) (36). The LC16m8 strain had a take rate of 95% (compared with 93.7% for Lister), fever rate of 7.7% (compared with 26.6% for Lister), and lower neurovirulence in a monkey assay. The lower fever rate and reduced neurovirulence were considered indications that this was a safer vaccine (37). Antibody titers and induration size were lower than those of the Lister strain; however, the effect of its decreased immunogenicity on the ability of this vaccine to protect against smallpox infection is unknown since the vaccine was never used in a smallpox-endemic region.

Similarly, the Modified *Vaccinia* Ankara was derived from the Ankara *Vaccinia* strain and is one of the most highly attenuated strains. With >570 passages in chicken embryo fibroblasts, it is host restricted and unable to replicate in human and other mammalian cells. Pock lesions did not form at the site of inoculation, and no adverse reactions were observed in clinical trials in persons at high risk with skin lesions (38). Modified *Vaccinia* Ankara was intended to serve as an attenuated smallpox vaccine for primary vaccinations in regions where smallpox was not endemic, since most of the adverse reactions associated with VACV occur after primary vaccination. The vaccine was safely used to vaccinate >120,000 persons in Turkey and Germany; however, its effectiveness against smallpox is unknown.

The cell substrate must be screened extensively for both endogenous and exogenous viral contamination. The measures taken to remove, inactivate, or prevent contamination of the product from any adventitious agent present in the cell substrate should be described. When applicable, carefully designed viral clearance studies should be conducted with different methods of virus inactivation or removal in the same production process to achieve maximum viral clearance. In addition, studies should be performed to assess virus inactivation and removal. The FDA has drafted several documents that provide guidance concerning testing biological products for adventitious agents (Appendix).

## Clinical Trials

### Phase 1

Phase 1 vaccine trials are primarily designed to evaluate safety and immunogenicity in small groups (e.g., 10 to 20 persons) of closely monitored healthy adult volunteers. Clinical evaluations of safety in Phase 1 studies should include both local injection site (lesion measurement) and systemic reactions, as well as hematologic testing, serum chemistries, and other laboratory studies. At predefined intervals, periodic assessments of the local injection site and systemic signs and symptoms would normally be recorded. For live attenuated VACV vaccines, active monitoring of the immunization site would be required for at least 21 days or until formation and separation of the scab, whichever is longer.

One difficulty in evaluating a new smallpox vaccine is demonstrating that the vaccine generates a protective immune response in the recipient. The appearance of a vaccine take or lesion is thought to be an important correlate of immunity. In Phase 1 trials, the frequency and size of the lesion generated by a new vaccine should be compared with the values observed historically for the current licensed calf lymph-type vaccine. It is also important to compare the breadth and scale of the hosts' immune response with responses generated by vaccines previously used against smallpox infection. One of the challenges will be developing validated assays to evaluate vaccine efficacy. In a Phase 1 trial, VACV-binding antibodies should be determined by ELISA and plaque neutralization assays. Extensive characterization of the immune response, including investigation of the cellular as well as the humoral response, can be pursued in Phase 2 studies.

Situations in prelicensure trials that may lead to safety problems for both vaccinees and their close contacts need to be anticipated. Contraindications for vaccinees include immune disorders, HIV infection, eczema, history of eczema, other skin conditions including burns, immunosuppressive therapy, malignancies, lymphomas and leukemias, and pregnancy. Vaccinees who have close contacts with these contraindications should also be excluded. Mechanisms to address rare serious VACV vaccine complications (e.g., availability of VACV immunoglobulin treatment) need to be addressed in the protocol.

For live attenuated VACV products, sponsors should describe their proposed procedures for containing the live biological material during their clinical studies and should provide data on the expected survival of the organism in the environment. Shedding of live vaccine organisms would need to be evaluated; isolation of volunteers early in clinical development may be necessary to assess the shedding of VACV and the potential for spread to contacts. The vaccination site needs to be covered at all times with a porous bandage until the scab has separated and the underlying skin has healed.

A dry, porous bandage is preferred to prevent the accumulation of perspiration around the inoculation site, which can increase the risk for secondary inoculation (9). Subjects should receive a dressing kit, including a medical waste bag in case the dressing should come off. Subjects and health-care professionals who may handle these dressings should be instructed on the importance of handwashing after contact with the site to prevent both self-inoculation of the virus and contact with the site by unvaccinated persons.

### Phase 2

In Phase 2 studies, generally more subjects are enrolled than in Phase 1 studies, and further data are provided on safety and immunogenicity. Phase 2 vaccine studies are often randomized and well controlled in design. One purpose of Phase 2 studies is to identify a preferred vaccine formulation, dose, and schedule for further clinical development in definitive safety and efficacy trials.

Clinical studies to compare the new vaccine with the licensed Dryvax vaccine would be done at this phase. Study size for a pivotal immunogenicity study would be based on statistical design to provide enough power to detect differences in "vaccine take," immune response, and safety (common local reactions) after inoculation by scarification, compared with the licensed Wyeth Dryvax. Both the humoral and cellular arms of the host response should be measured.

Studies to validate immune response assays should begin early in the drug development process. The goal is to have validated assays in place to assess critical immune responses before pivotal immunogenicity studies are initiated (39). At a minimum, seroconversion would have to be determined by plaque neutralization assays, VACV binding antibodies by ELISA, and cellular responses by cytotoxic T-lymphocyte response and lymphoproliferation assays.

### Phase 3 and Beyond

Conducting large-scale clinical endpoint efficacy field trials for a new smallpox vaccine cannot be planned at this time, in part because there are no longer any populations at risk for naturally occurring smallpox infection. For new vaccines based on vaccine take rate and the development of neutralizing antibody responses, pivotal comparative immunogenicity studies of the new compared with the licensed vaccines will likely form the basis of efficacy assessment. Studies in humans to evaluate the ability of a vaccine to protect against subsequent challenge with a live attenuated VACV vaccine might also be informative.

Large-scale trials are needed to provide safety data to support the license application, especially to evaluate less common serious adverse events. Randomized, well-controlled trials would provide the most informative safety data.

Plans should be defined for obtaining adequate safety data. Pediatric use could be critical during a bioterrorist event; however, identifying an appropriate pediatric study population for safety and immunogenicity evaluation presents challenges. Plans for pediatric clinical development should be discussed with the FDA (40,41). Finally, given the historical information on adverse events associated with VACV vaccination, a rigorous Phase 4 study commitment is expected (42).

## VACV Immunoglobulin Development

VACV immunoglobulin (VIG) is the only approved product currently available for treating complications of VACV vaccination. It is derived from the immunoglobulin fraction of plasma from persons who were immunized with VACV. The Red Cross initially obtained the product from the sera of hyperimmunized army recruits. The current supply of VIG is owned by the Department of Defense, which has provided some of this material to CDC for release in response to emergencies. VIG can be obtained from CDC to treat adverse reactions of VACV vaccine recipients, such as laboratory workers exposed to VACV or related *Orthopoxviridae*.

VIG is believed to be effective against certain complications of VACV vaccinations; it is recommended by the CDC's Advisory Committee on Immunization Practices for use in treating eczema vaccinatum, vaccinia necrosum, severe generalized VACV infections, VACV infections of the eyes (but not keratitis) or mouth, and VACV infections in the presence of other skin lesions such as burns, impetigo, varicella zoster, or poison ivy (43). No randomized controlled clinical trials have been performed to evaluate therapeutic efficacy in patients with VACV complications. However, a standard of care has developed based on data consisting of case series and anecdotal reports, as well as controlled data suggesting that VIG may modify VACV infection if administered concomitantly with vaccine.

Limited data from unblinded controlled studies support the efficacy of VIG in certain situations. In a trial conducted in Madras, India, 705 family contacts of 208 smallpox patients were randomized to receive smallpox vaccine or smallpox vaccine plus VIG as soon as possible after the index patient was admitted to the hospital. Smallpox developed in 5 of 326 contacts who received VIG compared with 21 of 379 controls, for a relative efficacy of 70% in preventing natural smallpox (44) ($p<0.05$, calculated by the first author).

The potential for VIG to prevent postvaccine encephalitis when administered prophylactically with vaccine was studied among Dutch military recruits in a double-blinded, randomized, placebo-controlled trial (45). More than 106,000 recruits were randomized to receive VIG plus smallpox vaccine or placebo plus smallpox vaccine. Three cases of VACV-associated encephalitis occurred in the VIG group compared with 13 cases of encephalitis in the placebo group ($p<0.05$, calculated by author).

Published case series of patients with severe VACV vaccination complications treated with VIG suggest that VIG lowered case-fatality rates and shortened the course of disease (20,46-52). Other trials have used antiviral agents in an attempt to treat complications (52-54), and these agents did not appear to have greater benefit than VIG. VIG is not considered to be effective in treating postvaccine encephalitis and is contraindicated for the treatment of vaccinal keratitis(55).

The recommended therapeutic dosage of VIG is 0.6 mL/kg intramuscularly, or 42 mL for a 70-kg adult; this dosage may be repeated as often as weekly. Such high intramuscular volume can be associated with trauma and possible nerve damage. Future development of VIG may include intravenous formulations to obviate these dose-related problems.

A more basic problem for the use of VIG is the availability of licensed product. The amount of VIG needed to respond

to the adverse events associated with a large-scale vaccination program cannot be manufactured from the currently available human sera.

## Future Considerations

An important benefit of the eradication of naturally occurring smallpox was the cessation of smallpox vaccination and the elimination of iatrogenic VACV vaccine adverse events. Growing concern about the U.S. population's vulnerability to a potential terrorist attack with biological weapons has led to strong political commitment to develop and stockpile new vaccines and other agents to respond to such an event (56). This response requires the development and manufacture of an effective vaccine, as well as products to treat the potential complications arising from a widespread vaccination program.

The evaluation of these products, especially ones that do not induce a vaccine take and induce an immune response that substantially differs from that induced by the currently licensed vaccine, may pose problems. Specifically, the usual measures of efficacy that require exposure to natural disease currently are not possible because the disease has been globally eradicated. In addition, definitive human challenge and protection studies with *Variola* would not be possible for ethical reasons.

In general, the issue of providing substantial evidence of efficacy when the traditional efficacy studies in humans cannot be done is of concern to the FDA. To address this issue, FDA has published and requested comments on a proposed rule intended to address certain efficacy issues for new agents to be used against lethal or permanently disabling toxic substances (57). The proposed rule attempts to define standards so that new drug and biological products developed to prevent serious or life-threatening conditions could be approved for marketing on the basis of evidence of effectiveness derived from appropriate studies in animals, without adequate and well-controlled efficacy studies in humans (21 CFR 314.126). For example, the wide host range of poxvirus viruses could potentially lead to the exploration of a monkeypox model to obtain supporting data. For vaccines, human safety and immunogenicity data would also be needed to support such approvals. The final rule, when published, would be expected to facilitate the development and licensing of certain new products to protect against biological warfare. This proposal would not apply if approval can be based on other standards in FDA regulations.

The future response to complications inherent in a new smallpox vaccine during a wide-scale vaccination program could entail a combination approach including VIG, antiviral drugs, and immune-based therapy involving humanized antibodies or fragments of antibodies produced in animals. Antiviral medications could be mass-produced, stored for long periods of time, and distributed quickly, if needed. Cidofovir, a DNA polymerase inhibitor developed for *Cytomegalovirus* retinitis, has been found to be active in preventing *Variola* infection in cultured cells and protects mice against lethal VACV challenge (58,59). In the long term, more products should be developed to protect the general population against adverse events due to VACV infection. Each product will pose unique scientific issues for evaluation and licensure.

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## Appendix

A. Examples of U.S. Food and Drug Administration and International Conference on Harmonization (ICH) Documents Relevant to the Manufacture and Product Quality of New Smallpox Vaccines

| Title | Date |
|---|---|
| FDA points to consider in the characterization of cell lines used to produce biologicals | 1993 |
| ICH guidance on quality of biotechnological/biological products: derivation and characterization of cell substrates used for production of biotechnological/ biological products | 1998 |
| ICH guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin | 1998 |
| FDA guidance for industry: content and format of chemistry, manufacturing and controls information and establishment description information for a vaccine or related product | 1999 |
| FDA draft guidance for industry: revised preventive measures to reduce the possible risk of transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD) by blood and blood products | 2001 |

To obtain these documents, connect to http://www.fda.gov/cber/guidelines.htm or call the FDA Office of Communication, Training and Manufacturers Assistance at 1-800-835-4709.)

B. Examples of ICH Documents Relevant to Clinical Testing of Vaccines

| Title | Date |
|---|---|
| Good clinical practice: Consolidated guideline | 1997 |
| General considerations for clinical trials | 1997 |
| Structure and content of clinical study reports | 1996 |
| Statistical principles for clinical trials (draft) | 1997 |

To obtain these documents, connect to http://www.fda.gov/cber/guidelines.htm or call the FDA Office of Communication, Training and Manufacturers Assistance at 1-800-835-4709.

C. U.S. Code of Federal Regulations (CFR) and U.S. Pharmacopeia (USP) Standards Applicable to Vaccines

| Issuance | Subject |
|---|---|
| 21 CFR 50 | Protection of Human Subjects |
| 21 CFR 56 | Institutional Review Boards |
| 21 CFR 58 | Good Laboratory Practices |
| 21 CFR 210, 211 | Good Manufacturing Practices |
| 21 CFR 312 | Investigational New Drug Applications (INDs) |
| 21 CFR 314.126 | Adequate and Well-Controlled Studies |
| 21 CFR 610 | General Biological Product Standards |
| 21 CFR 610.12 | Sterility Testing |
| 21 CFR 610.13 | Purity Testing |
| USP 24-(85) | Bacterial Endotoxin Test |
| USP 24-(1510) | Pyrogen Test |
| USP 24-(1171) | Sterility |

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# *Trichomonas vaginalis*, HIV, and African-Americans

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*Trichomonas vaginalis* may be emerging as one of the most important cofactors in amplifying HIV transmission, particularly in African-American communities of the United States. In a person co-infected with HIV, the pathology induced by *T. vaginalis* infection can increase HIV shedding. *Trichomonas* infection may also act to expand the portal of entry for HIV in an HIV-negative person. Studies from Africa have suggested that *T. vaginalis* infection may increase the rate of HIV transmission by approximately twofold. Available data indicate that *T. vaginalis* is highly prevalent among African-Americans in major urban centers of the United States and is often the most common sexually transmitted infection in black women. Even if *T. vaginalis* increases the risk of HIV transmission by a small amount, this could translate into an important amplifying effect since *Trichomonas* is so common. Substantial HIV transmission may be attributable to *T. vaginalis* in African-American communities of the United States.

*Trichomonas vaginalis* is a protozoan parasite transmitted principally through vaginal intercourse. Infection with the organism, while frequently asymptomatic, can cause vaginitis in women and urethritis in men. Despite a relative paucity of studies on the prevalence and incidence of trichomoniasis, recent publications suggest that *T. vaginalis* is one of the most common sexually transmitted infections (STIs) in the United States, with an estimated 5 million new cases occurring annually (1). Although the organism appears to be highly prevalent and has a widespread geographic distribution, *Trichomonas* has not been the focus of intensive study nor of active control programs. This neglect is likely a function of the relatively mild nature of the disease (2), the lack of effect on fertility, and the historic absence of association with adverse birth outcomes (although recent data suggest a possible causal role in low birth weight and prematurity [3]). However, *Trichomonas* may play a critical and underrecognized role in amplifying HIV transmission (4). We present the rationale to support the hypothesis that *T. vaginalis* may be an important cofactor in promoting the spread of HIV and, in some circumstances, may have a major impact on the epidemic dynamics of HIV in African-American communities.

## Biologic Rationale

### Expanding the Portals of Entry and Exit

*T. vaginalis* infection typically elicits an aggressive local cellular immune response with inflammation of the vaginal epithelium and exocervix in women and the urethra of men (5). This inflammatory response induces a large infiltration of leukocytes, including HIV target cells such as CD4+ bearing lymphocytes and macrophages to which HIV can bind and gain access (6,7). In addition, *T. vaginalis* can frequently cause punctate mucosal hemorrhages (8). In an HIV-negative person, both the leukocyte infiltration and genital lesions induced by *Trichomonas* may enlarge the portal of entry for HIV by increasing the number of target cells for the virus and allowing direct viral access to the bloodstream through open lesions. Similarly, in an HIV-infected person the hemorrhages and inflammation can increase the level of virus-laden body fluids, the numbers of HIV-infected lymphocytes and macrophages present in the genital contact area, or both. The resulting increase of both free virus and virus-infected leukocytes can expand the portal of exit, thereby heightening the probability of HIV exposure and transmission to an uninfected partner. Increased cervical shedding of HIV has been shown to be associated with cervical inflammation (9), and substantially increased urethral viral loads have been documented in men with *Trichomonas* infection (10). In addition, *T. vaginalis* has the capacity to degrade secretory leukocyte protease inhibitor, a product known to block HIV cell attachment; this phenomenon may also promote HIV transmission (11). Moreover, since most patients with *Trichomonas* infection are asymptomatic or mildly symptomatic (12), they are likely to continue to remain sexually active in spite of infection. Studies suggest that approximately 50%-70% of persons with *T. vaginalis* have subclinical infection (12).

## Empiric Evidence Implicating Trichomonas in HIV Transmission

Data from studies conducted in Africa have shown an association between *Trichomonas* and HIV infection, suggesting a two- to threefold increase in HIV transmission

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(4,13,14). A cross-sectional study conducted among 1,209 female sex workers in the Ivory Coast found an association between HIV and *Trichomonas* infection in bivariate analysis (crude odds ratio 1.8, 95% confidence intervals 1.3, 2.7). In another cross-sectional study performed in Tanzania among 359 women admitted to a hospital for gynecologic conditions, *Trichomonas* was more common in women with HIV infection in multivariate analysis (odds ratio 2.96, no confidence intervals provided, $p<0.001$). While such cross-sectional studies are limited by the issue of temporal ambiguity, i.e., lack of information on whether *Trichomonas* infection preceded HIV, these preliminary findings were subsequently reinforced in a single prospective study from Zaire (4). This study, in which 431 HIV-negative female prostitutes were evaluated over time, found that prior *Trichomonas* infection was associated with a twofold increased rate of HIV seroconversion in muiltivariate analysis.

## Data on the Prevalence of *T. vaginalis* among U.S. Women

Information on the occurrence of *T. vaginalis* infection in the United States is meager. Trichomoniasis is not a reportable condition in most health jurisdictions, and prevalence surveys for STIs often do not include attempts to recover *Trichomonas*. In addition, the relatively few published studies with information on the prevalence of *T. vaginalis* infection have generally been conducted among highly selected populations, typically included only women, or were limited by small numbers of participants. Frequently these studies were not conducted with the primary purpose of assessing the prevalence of *Trichomonas*. Moreover, many of these studies have often used diagnostic techniques with relatively low sensitivity such as wet mount, stained preparations, or Papanicolaou (PAP) smear. Wet mount, the most commonly used method, has an estimated sensitivity of 58% when compared with culture (15); the sensitivity of PAP smear is approximately 57%. The accuracy of these techniques is dependent on the experience of the microscopist, and sensitivities may vary widely (15). The sensitivity of culture when compared with polymerase chain reaction (PCR) has been estimated to be 70% (16). Such highly sensitive PCR and related techniques are not routinely used nor readily available for *Trichomonas* as for other STIs (17). As a result of suboptimal laboratory methods, studies of *T. vaginalis* have often substantially underestimated the prevalence of infection. In spite of this, levels of infection have typically been high, with reported overall prevalences ranging from 3% to 58% and an unweighted average across studies of 21% (18-37).

Table 1 lists published reports on the occurrence of *T. vaginalis* infection among women conducted among U.S. populations from 1964 through 1999. Although not

Table 1. Studies of the prevalence of *Trichomonas vaginalis* infection in women, United States, 1964–1997

| Year[a] | Location (ref) | N | Population | Trichomonas prevalence (%) | Diagnostic method(s) |
|---|---|---|---|---|---|
| 1996-97 | New York (18) | 213 | Incarcerated | 47 | culture |
| 1995-97 | St. Louis (19) | 143 | HIV clinic | 11 | wet mount |
| 1993-95 | 4 cities (20) | 1,285 | HIV infected and high risk | 11 | wet mount |
| 1994 | New York (23) | 1,404 | Inner city | 20 | not provided |
| 1992 | Baltimore (24) | 279 | STD clinic | 26 | culture |
| 1990-94 | New York (37) | 677 | HIV and community clinics | 22 | culture |
| 1901-93 | Southeastern city (21) | 650 | Adolescent health clinics | 3 | culture |
| 1986 | 5 cities (27) | 13,816 | Antepartum women | 13 | culture |
| 1990-91 | New York (22) | 372 | Inner city | 27 | culture |
| 1989-90 | New York (25) | 1,401 | OB/GYN clinics | 20 | culture |
| 1989 | Baltimore (26) | 3,005 | Cancer screening | 25 | wet mount |
| 1987-88 | Denver (36) | 5,681[b] | STD clinic | 11 | wet mount |
| 1984-86 | Birmingham (28) | 818 | STD clinic | 21 | wet mount |
| 1985 | San Francisco (29) | 171 | Adolescent clinic | 11 | wet mount/PAP[c] |
| 1982 | Baltimore (30) | 115 | Pregnant adolescents | 34 | culture |
| 1981 | Seattle (31) | 80 | Juvenile detention | 48 | wet mount |
| 1980 | Providence (32) | 500 | Student health center | 3 | culture |
| 1979-80 | Storrs (33) | 383 | GYN clinic | 19 | wet mount/PAP |
| 1971 | Oregon (34) | 338 | State school/adolescents | 35 | Gram stain |
| 1964 | Philadelphia (35) | 27,392 | Cancer screening | 16 | PAP |

[a]Year of study (or publication).

[b] Number of visits.

[c]Papanicolaou smear; STD = sexually transmitted disease; OB/GYN = obstetrics/gynecology.

necessarily complete, a comprehensive search through MEDLINE and review of articles yielded only 20 reports during this 35-year period. Evaluated populations have included such groups as sexually transmitted disease (STD) clinic patients, inner-city populations, pregnant women, university students, adolescents, incarcerated populations, and women with HIV infection.

### Data on the Incidence of *T. vaginalis* in the United States

Even fewer studies have assessed the incidence of trichomoniasis in the United States. In a study conducted from 1992 to 1995 among a cohort of 212 women with HIV in Los Angeles County, *Trichomonas* infection was the most frequently identified sexually transmitted disease and was found in 37 (17.4%) women, representing a crude incidence rate of 14.1 per 100 person-years' experience (38). The crude rate was highest in black women (69.0 per 100 person-years). A recent prospective study conducted from 1990 to 1998 in New Orleans, which followed women co-infected with HIV and *T.* vaginalis, documented high rates (16.1 per 100 person-years) of *Trichomonas* re-infection (39). Among a predominantly black group of HIV-infected and high-risk women followed in New York City from 1990 to 1994, *T. vaginalis* was the most frequent incident STI (37).

### Prevalence of *T. vaginalis* among Men in the United States

Very few published studies have assessed the prevalence of *T. vaginalis* among men and, as is the case for women, these studies typically have included relatively small samples from selected populations. Often data on race-specific prevalences are not provided. Among men attending an STD clinic in Seattle-King County from 1987 to 1990, 6% of 300 randomly selected men were infected with *Trichomonas* by culture technique; 22% of 147 contacts to women with *T. vaginalis* were also positive (40). In a study published in 1995 conducted in Richmond, California, 12% of 204 male patients from an STD clinic were culture positive for *T. vaginalis* (41). Among 454 consecutive men attending an STD clinic in Denver in 1998, 2.8% were found to be infected by a culture method (42). In a small-scale study published in 1991 among 16- to 22-year-old black men enrolled in an inner-city residential youth job-training program, *Trichomonas* was recovered from 55% of 85 participants and was the most common STI identified (43). Data on race-specific prevalences of *Trichomonas* infection among U.S. males are not available. We are unaware of any published reports that have assessed the prevalence of *T. vaginalis* in males and females. While the separate studies we have cited suggest that *Trichomonas* may be more common in women in the United States, the data are so limited and potentially biased that any such conclusions must be made cautiously.

### Race and *Trichomonas*

Table 2 presents data, where available, on the prevalence of *Trichomonas* among women, by race, in the United States. In each study that has presented information on race/ethnicity, the prevalence of *Trichomonas* has been highest in African-Americans (23%-51%), ranging from approximately 1.5 to nearly 4 times greater than other racial/ethnic groups. In several studies in which very high prevalences of infection were observed, the population consisted exclusively or predominantly of African-Americans. This racial finding, consistent across studies, is unlikely to be artifactual.

Table 2. Prevalence of *Trichomonas vaginalis* among women, by race, United States

| City (ref) | Overall *Trichomonas* prevalence (%) | *Trichomonas* prevalence in blacks | *Trichomonas* prevalence in non-blacks | OR[a] |
|---|---|---|---|---|
| New York (18) | 47 | 51 | 35 | 1.6 |
| San Francisco (29) | 11 | 28 | 9 | 3.7 |
| 5 cities (27) | 13 | 23 | 6 | 4.4 |
| Philadelphia (35) | 16 | 30 | 11 | 3.6 |
| New York (22) | 27 | population 92% black | | |
| New York (25) | 20 | population 83% black | | |
| Baltimore (24) | 26 | population 96% black | | |
| New York (23) | 20 | population 90% black | | |
| Baltimore (26) | 25 | population 100% black | | |
| Birmingham (28) | 21 | population 89% black | | |
| Providence (32) | 3 | population 87% black | | |

[a] Estimated odds ratio.

Several factors may explain the apparent elevated rate of trichomoniasis in black women. This phenomenon may indicate a high prevalence of *Trichomonas* infection among the sex partners of these women. Although a study in Washington, D.C., observed a high prevalence of *T. vaginalis* (55%) among young, inner-city, black men (43), data on race-specific rates of *Trichomonas* infection in men are lacking. The association with black race may also reflect decreased use of barrier protection in this population. Studies indicate that African-American males are less likely to use condoms than men of other racial groups because of a higher frequency of condom breakage and slippage (44) and a reported decrease in sexual fulfillment (45). Alternatively, it is possible that practices such as douching, which is reportedly more common in black women (46) and can increase susceptibility to other STIs (47), could predispose to trichomoniasis and explain the observed racial association. Increased prevalences of *Trichomonas* infection could also reflect lack of access to care and distrust of the health-care system, which could manifest as failure to seek care, noncompliance with treatment recommendations, and hesitation to refer partners for treatment. Drug use and its association with high-risk sexual behaviors, including trading sex for money or drugs, may also explain the racial differences in the occurrence of *Trichomonas*. In addition, compared with other racial and ethnic groups, a greater proportion of blacks are unmarried, divorced, or separated (48), and unmarried status is itself a risk marker for STIs (49). It is also conceivable that a genetic or racial-based heightened susceptibility to *T.vaginalis* exists in African-Americans; however, such a

phenomenon has not been recognized. Finally, the observed racial disparity could reflect strain differences of *Trichomonas*. For example, if the strains that infect African-Americans are more likely to produce chronic, persistent infection of longer duration, higher prevalences would be observed. However, this hypothesis has not been studied.

### *Trichomonas* Compared with Other STIs in African-American Women

Table 3 lists studies comparing the prevalence of *T. vaginalis* infection with that of other STIs among black women in the United States. In each study *Trichomonas* was the most commonly identified STI, exceeding both *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in prevalence. While the optimal tests for detecting *C. trachomatis* and *N. gonorrhoeae* were not always used in these studies, neither were highly sensitive tests used for the diagnosis of *Trichomonas*.

### Discussion and Implications

The HIV/AIDS epidemic is a heterogeneous one, impacting communities and subpopulations in disproportionate ways. In many jurisdictions in the United States, HIV is increasingly affecting low-income groups, particularly African-Americans and women. We suggest that part of this phenomenon may result from the amplifying effect of *T.vaginalis*. Several aspects of the biology and epidemiology of *Trichomonas* suggest that this long-neglected protozoan may play an important role in HIV transmission dynamics. A compelling biologic rationale suggests that the pathology caused by *Trichomonas* enhances the efficiency of HIV transmission. In addition, *T. vaginalis* infection is often asymptomatic, and affected persons are likely to continue to engage in sexual activity. This strong biologic plausibility is supported by empiric studies from Africa documenting that *Trichomonas* may increase HIV transmission by two- to threefold. Moreover, although imperfect, the available data suggest that *T. vaginalis* is a highly prevalent infection, particularly among African-American women in urban communities of the United States. Given the evidence that *T.vaginalis* likely promotes HIV infection, the apparent high level of *Trichomonas* infection in black women is cause for concern. Even if *T. vaginalis* increases the risk of HIV transmission by a small or modest amount, it may translate into a sizable population effect since *Trichomonas* is so common. To illustrate this, we present population-attributable risk curves, or the level of HIV transmission that would be attributable to *T. vaginalis*, at varying prevalences of *Trichomonas,* given the assumption of an increased relative risk of HIV infection of 2 or 3 (Figure). As the figure illustrates, if *Trichomonas* amplifies HIV transmission by twofold and the prevalence of *T. vaginalis* in a community is 25%, one fifth (20%) of HIV transmission in that population would be attributable to *Trichomonas*. This has important implications for HIV prevention. Reduction in the prevalence of *Trichomonas* could translate into substantial decreases in HIV transmission. Effective, inexpensive single-dose therapy (2 g oral metronidazole) is available for the treatment of *T. vaginalis* infection. It may not be hyperbole to suggest that *Trichomonas* infection may be more readily modifiable than sexual behavior in some high-risk groups. Trials in Tanzania have demonstrated the benefit of reduced HIV incidence in communities receiving aggressive STD control intervention (50).

While convincing data suggest that other STDs, including both ulcerative and inflammatory infections, promote HIV transmission (51), available evidence suggests that *T.vaginalis* is the most common STI in African-American women and therefore may play a more prominent role than other STIs in augmenting the spread of HIV in this high-risk group.

Additional studies to evaluate the prevalence and incidence of *T. vaginalis* and to determine risk factors for infection in both men and women are needed. Moreover, given the paucity of data and the potential importance of *Trichomonas*, consideration should be given to requiring mandatory reporting of *T. vaginalis* infection. Efforts to further evaluate

Table 3. Studies comparing the prevalence of *Trichomonas vaginalis* infection with that of other sexually transmitted infections among black women in the United States

| Year | City (ref) | Trichomonas (%) | Chlamydia (%) | Gonorrhea (%) |
|---|---|---|---|---|
| 1996 | New York (18) | 51 | 9 | 5 |
| 1994 | New York (22) | 27 | 7 | 2 |
| 1994 | New York (23) | 20 | 15 | no data |
| 1992 | Baltimore (24) | 26 | 21 | 14 |
| 1990 -94 | New York (37) | 22 | 6 | 1 |
| 1985 | San Francisco (29) | 28 | 25 | no data |

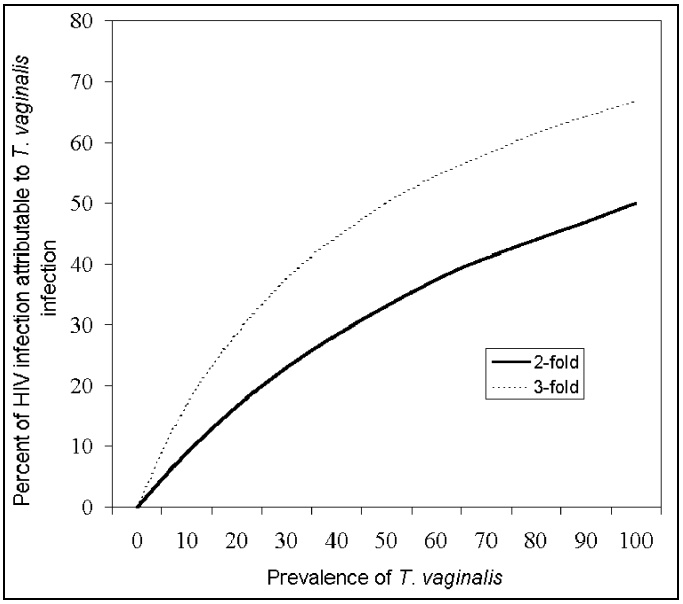


Figure. Hypothetical level of HIV transmission attributable to *Trichomonas vaginalis* at varying prevalences of *Trichomonas* infection and assuming that *T. vaginalis* infection amplifies HIV infection by two- or three-fold.

the interactions between *T. vaginalis* and HIV, particularly in an industrialized country setting, would also seem warranted. However, given the lower rates of heterosexual transmission, such studies would be expensive and require a large sample. Nevertheless, we believe that current information is compelling enough to warrant considering implementation of efforts to identify and treat persons with *T. vaginalis* infection, particularly African-Americans, in areas of overlapping HIV and *T. vaginalis* epidemics. Screening programs using self-collected vaginal swabs (52) for culture may be a reasonable method for such an effort. An alternative approach would be to first use wet mount examination, which is relatively easy and inexpensive but lacks sensitivity, followed by culture for specimens that are negative on wet mount. Recent development of sensitive and specific urine-based diagnostic techniques can enhance both the yield and ease of screening efforts (53); however, issues of cost and accessibility may limit the use of such methods for the average physician.

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# Bioterrorism-Related Inhalational Anthrax: The First 10 Cases Reported in the United States

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From October 4 to November 2, 2001, the first 10 confirmed cases of inhalational anthrax caused by intentional release of *Bacillus anthracis* were identified in the United States. Epidemiologic investigation indicated that the outbreak, in the District of Columbia, Florida, New Jersey, and New York, resulted from intentional delivery of *B. anthracis* spores through mailed letters or packages. We describe the clinical presentation and course of these cases of bioterrorism-related inhalational anthrax. The median age of patients was 56 years (range 43 to 73 years), 70% were male, and except for one, all were known or believed to have processed, handled, or received letters containing *B. anthracis* spores. The median incubation period from the time of exposure to onset of symptoms, when known (n=6), was 4 days (range 4-6 days). Symptoms at initial presentation included fever or chills (n=10), sweats (n=7), fatigue or malaise (n=10), minimal or nonproductive cough (n=9), dyspnea (n=8), and nausea or vomiting (n=9). The median white blood cell count was 9.8 X $10^3$ /$mm^3$ (range 7.5 to 13.3), often with increased neutrophils and band forms. Nine patients had elevated serum transaminase levels, and six were hypoxic. All 10 patients had abnormal chest X-rays; abnormalities included infiltrates (n=7), pleural effusion (n=8), and mediastinal widening (seven patients). Computed tomography of the chest was performed on eight patients, and mediastinal lymphadenopathy was present in seven. With multidrug antibiotic regimens and supportive care, survival of patients (60%) was markedly higher (<15%) than previously reported.

Historically, human anthrax in its various forms has been a disease of those with close contact to animals or animal products contaminated with *Bacillus anthracis* spores. In the mid-1800s, inhalational anthrax related to the textile industry became known as woolsorters' disease (in England) (1) and ragpickers' disease (in Germany and Austria) because of the frequency of infection in mill workers exposed to imported animal fibers contaminated with *B. anthracis* spores. In the early 1900s, human cases of inhalational anthrax occurred in the United States in conjunction with the textile and tanning industries. In the last part of the 20th century, with improved industrial hygiene practices and restrictions on imported animal products, the number of cases fell dramatically (1,2); however, death rates remained high (>85%) (1,3). In 1979, in Sverdlovsk, former Soviet Union, an apparent aerosol release of *B. anthracis* spores from a military facility resulted in the largest outbreak of inhalational anthrax in the 20th century (3).

Before October 2001, the last case of inhalational anthrax in the United States had occurred in 1976 (1,4).

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Identification of inhalational anthrax in a journalist in Florida on October 4, 2001, marked the beginning of the first confirmed outbreak associated with intentional anthrax release in the United States (5,6). We describe the clinical presentation of the first 10 cases of this bioterrorism-related outbreak.

## Methods

From October 4 to November 2, 2001, the Centers for Disease Control and Prevention (CDC) and state and local public health authorities reported 10 confirmed cases of inhalational anthrax and 12 confirmed or suspected cases of cutaneous anthrax in persons who worked in the District of Columbia, Florida, New Jersey, and New York (6,7). Epidemiologic investigation indicated that the outbreak resulted from intentional delivery of *B. anthracis* spores through mailed letters or packages.

Of the 10 inhalational cases, 7 occurred in postal employees in New Jersey and the District of Columbia who were likely exposed to letters known to be contaminated with *B. anthracis* spores. Two cases were in employees of a media company in Florida: one is believed to have received contaminated mail, the other to have sorted and distributed that mail. Case 10 was in a resident of New York, and the nature of her exposure to *B. anthracis* is currently unknown.

In the course of the US Public Health Service response to the outbreak, CDC, in conjunction with state and local health departments, collected clinical data from patients with confirmed inhalational anthrax as defined by the CDC surveillance case definition (8). Data were collected through a variety of methods, including interviews with patients and family members, physical examinations of patients, discussions with clinicians, reviews of medical records, and analyses of clinical and pathologic specimens.

Presumptive isolation of *B. anthracis* in these patients was confirmed by gamma phage lysis, presence of a capsule, detection of capsule and cell-wall antigens by direct fluorescent antibody, and *B. anthracis*-specific polymerase chain reaction (PCR). All isolates were confirmed by state and other laboratory facilities of the National Bioterrorism Laboratory Response Network and by the laboratories of the National Center for Infectious Diseases (NCID), CDC. Other tests performed at NCID for confirming the diagnosis of inhalational anthrax (8) included immunohistochemical testing of clinical specimens by using *B. anthracis* capsule and cell-wall antibody, *B. anthracis*-specific PCR, and serologic detection of immunoglobulin (Ig) G to *B. anthracis* protective antigen (PA).

## Case Descriptions

### Case 1

On October 2, 2001, a 63-year-old Caucasian photo editor working for a Florida newspaper awoke early with nausea, vomiting, and confusion and was taken to a local emergency room for evaluation. His illness, which started on September 27 during a trip to North Carolina, was characterized by malaise, fatigue, fever, chills, anorexia, and sweats. No history of headache, cough, chest pain, myalgias, dyspnea, abdominal pain, diarrhea, or skin lesions was reported. Past medical history included hypertension, cardiovascular disease, and gout. He did not smoke. On admission, the patient was alert and interactive but spoke nonsensically. Temperature was 39.2°C, heart rate 109/min; blood pressure and respiratory rate were normal. Initial pulmonary, heart, and abdominal examinations were reported as normal. No nuchal rigidity was observed. He was not oriented to person, place, or time. Admission laboratory values included a normal total white blood cell (WBC) count, but the platelet count was low. Serum chemistries were normal, except for borderline hyponatremia and elevated total bilirubin. He had mild metabolic acidosis (Table 1). A chest X-ray showed a prominent superior mediastinum and a possible small left pleural effusion (Figure 1). Cerebrospinal fluid (CSF) analysis showed WBC count 4,750/µL (81% neutrophils), red blood cell count 1,375/µL, glucose 57 mg/dL (serum glucose 174 mg/dL), and protein 666 mg/dL. Microscopy examination of the CSF showed many gram-positive bacilli (Figure 2). *B. anthracis* was isolated from CSF after 7 hours of incubation and from blood cultures within 24 hours of incubation. The patient was admitted to the hospital with a diagnosis of meningitis. After a single dose of cefotaxime, he was started on multiple antibiotics, including ceftazidime, gentamicin, metronidazole, doxycycline, ampicillin, and trimethoprim-sulfamethoxazole. Shortly after admission, he had generalized seizures and was intubated for airway protection. On hospital day 2, penicillin G, levofloxacin, and clindamycin were begun; ampicillin, ceftazidime, and trimethoprim-sulfamethoxazole were discontinued. He remained febrile and became unresponsive to deep stimuli. His condition progressively deteriorated, with hypotension and worsening renal insufficiency. The patient died on October 5. Autopsy findings included hemorrhagic mediastinal lymphadenitis, and immunohistochemical staining showed disseminated *B. anthracis* in multiple organs.

### Case 2

On September 24, a 73-year-old Hispanic man, the newspaper mailroom clerk who delivered mail to the patient in Case 1, had onset of fatigue. On September 28, nonproductive cough, intermittent fever, rhinorrhea, and conjunctivitis developed. From September 28 to October 1, he had gradual progression of cough, marked worsening of fatigue with lethargy, onset of exertional dyspnea, fever, and sweats. He had mild abdominal pain associated with vomiting, and his coworkers and family noted intermittent periods of confusion. He had no underlying chronic illnesses, with the exception of a transient ischemic attack in August 2001. He did not smoke. He was admitted to the hospital on October 1. Temperature was 38.5°C, heart rate 109/min, respiratory rate 20/min, and blood pressure 108/61 mm Hg. He had bilateral conjunctival injection and bilateral pulmonary rhonchi. Examination, including assessment of neurologic function, was otherwise unremarkable. No skin lesions were observed. Admission laboratory results included normal WBC count and serum chemistries, except for hypoalbuminemia, elevated hepatic transaminases, borderline hyponatremia, and increased creatinine. Arterial blood gas values showed hypoxia (Table 1). Blood cultures obtained on hospital day 2, after initiation of antibiotics, showed no growth. A chest X-ray showed left upper and lower lobe infiltrates consistent with pneumonia and a small left pleural effusion (Figure 3).

Table 1. Laboratory findings at initial visit to health-care provider for 10 patients with bioterrorism-related inhalational anthrax, October through November 2001

| | Case 1 | Case 2 | Case 3 | Case 4 | Case 5 | Case 6 | Case 7 | Case 8 | Case 9[a] | Case 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| WBC/mm3[b] | 9,400 | 9,900 | 7,500 | 9,700 | 10,300 | 13,300 | 9,700 | 8,100 | 11,200 | 11,400 |
| WBC differential | | | | | | | | | | |
| Neutrophils % | 76 | 72 | 76 | 43 | 83 | 78 | 79 | 50 | 67 | 83 |
| Band forms % | ND | ND | 8 | 10 | 4 | ND | ND | 6 | 14 | ND |
| Lymphocytes% | 15 | 10 | 7 | 24 | 6 | 11 | 14 | 30 | 8 | 9 |
| Monocytes% | 9 | 17 | 7 | 15 | 7 | 8 | 7 | 10 | 11 | 7 |
| Hematocrit % | 45.7 | 47.1 | 46.9 | 48.6 | 43.0 | 51.4 | 44.7 | 45.3 | 42.5 | 46.3 |
| Platelet count x 103/ mm3 | 109 | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL | WNL |
| Sodium mmol/l | 132 | WNL | WNL | WNL | ND | WNL | 134 | 133 | 133 | 134 |
| Potassium mmol/l | WNL | WNL | WNL | WNL | ND | WNL | 3.4 | WNL | WNL | WNL |
| Chloride mmol/l | WNL | WNL | WNL | WNL | ND | WNL | WNL | 98 | WNL | WNL |
| Bicarbonate mmol/L | WNL | WNL | WNL | WNL | ND | WNL | 20 | WNL | WNL | WNL |
| Creatinine mg/dL | WNL | 1.2 | WNL | WNL | ND | WNL | WNL | WNL | WNL | WNL |
| SGOT IU/L | WNL | 405 | 106 | 153 | ND | WNL | ND | 269 | 130 | 240 |
| SGPT IU/L | WNL | 408 | 123 | 184 | ND | 44 | ND | 168 | 120 | 263 |
| Alkaline phosphatase IU/L | WNL | 141 | 165 | 171 | ND | WNL | ND | WNL | 147 | WNL |
| Albumin g/dL | WNL | 2.3 | 2.9 | 2.9 | ND | WNL | ND | WNL | 3.4 | 3.3 |
| Bilirubin mg/dL | 1.5 | WNL | 1.9 | 1.7 | ND | WNL | ND | WNL | WNL | WNL |
| Arterial pH | 7.32 | 7.48 | 7.45 | 7.46 | ND | ND | ND | 7.51 | ND | 7.41 |
| Arterial PaCO2 mm Hg | 37 | 33 | 27 | 38 | ND | ND | ND | 26 | ND | 40 |
| Arterial PaO2 mm Hg (FiO2) | 208 (70%) | 52 (RA) | 80 (RA) | 66 (RA) | ND | ND | ND | 58 (RA) | ND | 122 (100% NRBR) |
| Pleural fluid RBC/mm3 | ND | 110,000 | 243,586 | 179,841 | ND | ND | ND | 80,000 | "Many" | 73,000 |
| WBC/mm3 (% neutrophils) | ND | 2,260 (58%) | 1,707 (15) | 1,633 (6) | ND | ND | ND | 785 (12) | 286 (71) | 3,000 (90) |
| Protein g/dL | ND | 2.6 | 2.7 | 4.3 | ND | ND | 2.01 | 4.8 | 4.1 | 4.2 |
| LDH IU/L | ND | 282 | 1,762 | 965 | ND | ND | 261 | 319 | 740 | 1,264 |

[a] The patient in Case 9 was initially seen as an outpatient 2 days before hospital admission, but had no laboratory studies performed at that time. These results were obtained from studies done on the day of admission.

[b] WBC = white blood cells; WNL = within normal limits; ND = not done; NA = not available; SGOT = serum glutamic oxalacetic transaminase; SGPT = serum glutamic pyruvic transaminase; $FiO_2$ = fraction of inspired $O_2$; RA = room air; NRBR = nonrebreather mask; RBC = red blood cells; LDH = lactate dehydrogenase

No mediastinal widening was observed. The patient was initially given intravenous azithromycin; cefotaxime and ciprofloxacin were subsequently added. A nasal swab obtained on October 5 grew B. anthracis. Computed tomography (CT) of the chest showed bilateral effusions and multilobar pulmonary consolidation but no significant mediastinal lymphadenopathy (Figure 4). A left thoracentesis yielded serosanguinous fluid (Table 1) positive for *B. anthracis* DNA by PCR. Bronchoscopy showed bloody secretions in the right lower lobe and left lung, with severe mucosal hyperemia, mottling, and inflammation. Bacterial cultures of bronchial washings and pleural fluid did not grow. A transbronchial biopsy showed *B. anthracis* capsule and cell-wall antigens by immunohistochemical staining. Tests for *Legionella* spp., acid-fast bacteria, *Pneumocystis carinii*, *Chlamydia* spp., *Leptospira*, and *Hantavirus* and other viral pathogens were negative. The hospital course included an episode of supraventricular tachycardia with hypotension, maximum WBC count 26,800/ $mm^3$, and recurrent left pleural effusion that required repeat thoracentesis and placement of a chest tube. The pleural fluid from the second thoracentesis was positive for *B. anthracis* DNA by PCR. A pleural fluid cytology preparation and pleural biopsy showed *B. anthracis* capsule and cell-wall antigens by immu-

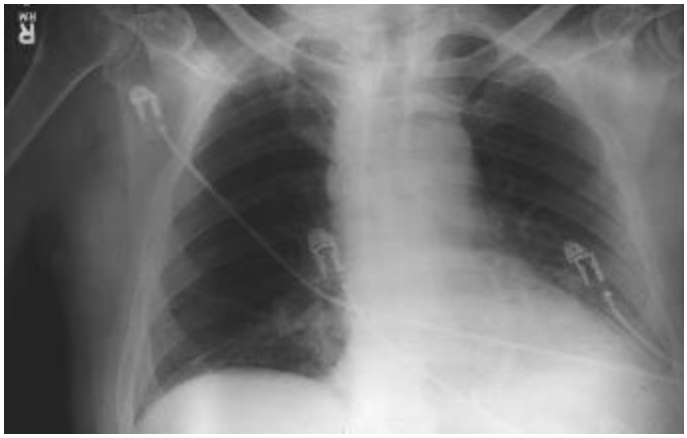

Figure 1. Initial chest X-ray (Case 1) showing prominent superior mediastinum and possible small left pleural effusion.

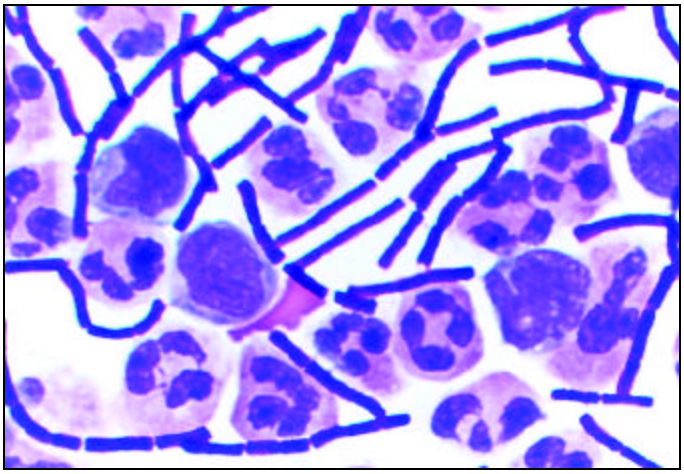

Figure 2. Gram stain of cerebrospinal fluid (Case 1) showing *B. anthracis*.

nohistochemical staining. In addition, serial serum samples demonstrated a >4-fold rise in levels of serum antibody (IgG) to the PA component of the anthrax toxins by enzyme-linked immunosorbent assay (ELISA). The patient's condition gradually improved, and he was discharged from the hospital October 23 on oral ciprofloxacin.

### Case 3

On October 16, a 56-year-old African-American man, a US Postal Service mail sorter, noted low-grade fever, chills, sore throat, headache, and malaise. This was followed by minimal dry cough, chest heaviness, shortness of breath, night sweats, nausea, and vomiting. On October 19, when he arrived at a local hospital, he was afebrile and normotensive. Heart rate was 110/min, and he was not tachypneic. He was in no acute distress but had decreased breath sounds and rhonchi at the left base. No skin lesions were observed. His past medical history was unremarkable, and he did not smoke. Total WBC count was normal, but there was a slight left shift in the differential. Hepatic transaminase levels and bilirubin were elevated. Serum albumin was decreased, but serum chemistries and renal function were normal. Arterial blood gas values showed adequate oxygenation (Table 1). A chest X-ray showed a widened mediastinum (especially in the right paratracheal region), bilateral hilar masses, bilateral pleural effusions, and a small right lower lobe air space opacity. CT of the chest showed diffuse mediastinal edema; marked paratracheal, subcarinal, hilar, and azygo-esophageal recess adenopathy (the largest node measuring 4.2 cm in diameter); and bilateral moderate pleural effusions. Within 11 hours, admission blood cultures grew *B. anthracis*. Ciprofloxacin, rifampin, and clindamycin were initiated. On October 21, the patient had respiratory distress, which was treated with diuretics, systemic corticosteroids, and a therapeutic thoracentesis. During the course of hospitalization, the pleural effusion reaccumulated, necessitating two additional thoracenteses. All three pleural fluid specimens were hemorrhagic. The patient did not require a chest tube. Hematemesis developed, and several shallow gastric ulcers were noted on upper endoscopy. On October 28, the patient had signs of hemolytic anemia and thrombocytopenia, which was treated with plasmapheresis. Hematologic values subsequently improved, and the patient remains hospitalized in stable condition.

### Case 4

On October 16, a 56-year-old African-American man, a US Postal Service worker, noted mild headache that was constant and global but not associated with visual changes, stiff neck, or other neurologic symptoms. Over the following 3 days, the headache worsened and was accompanied by low-grade fever, chills, sore throat, myalgias, nausea, malaise,

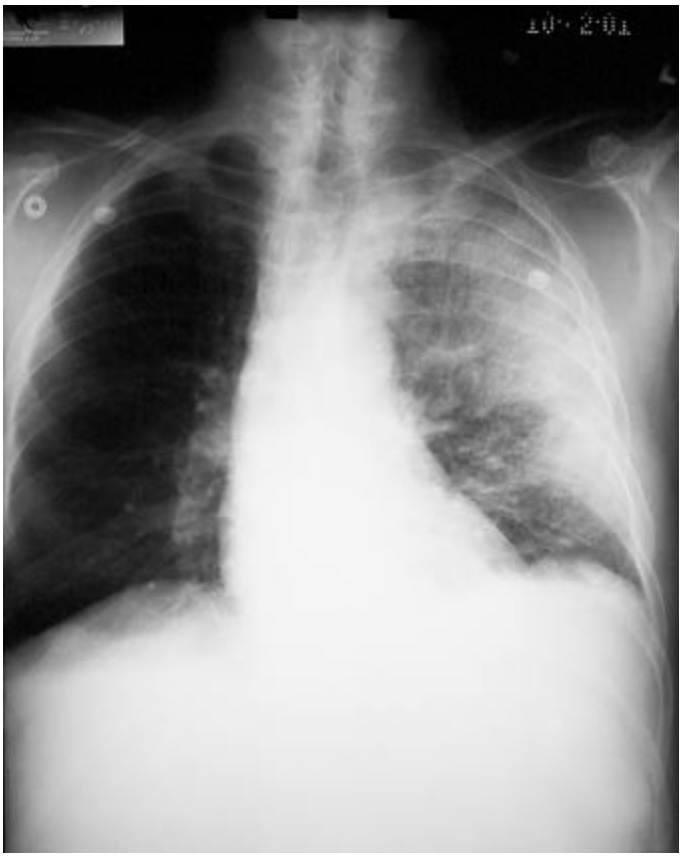

Figure 3. Chest X-ray (Case 2) showing diffuse consolidation consistent with pneumonia throughout the left lung. There is no evidence of mediastinal widening.

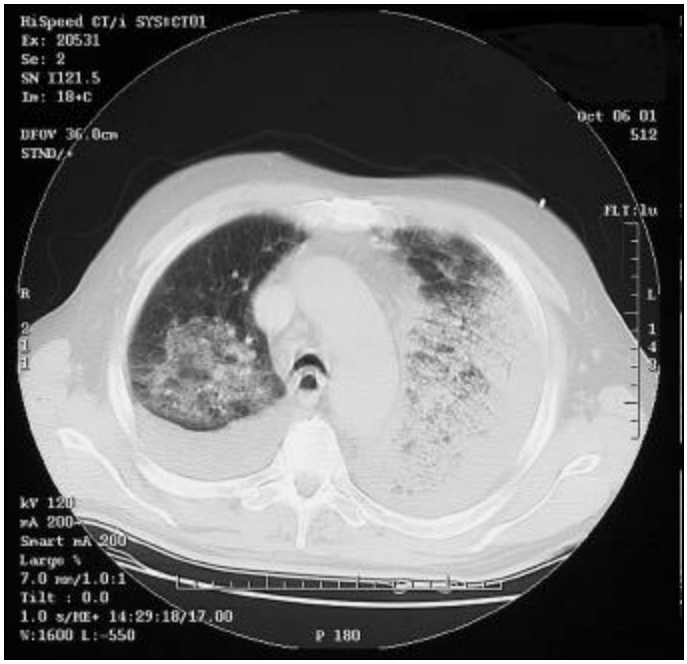


Figure 4. Computed tomography of chest (Case 2) showing bilateral pulmonary consolidation and pleural effusions.

drenching sweats, intermittent blurred vision, and photophobia. A mild dry cough, dyspnea on exertion, and pleuritic chest pain developed. When he arrived at the hospital on October 20, he was afebrile and normotensive, pulse was 127/min, and respiratory rate 20/min. He was in no acute distress, and physical examination was unremarkable, except for decreased breath sounds at both bases. His past medical history was unremarkable, and he did not smoke. Admission laboratory results were normal, except for elevated bilirubin and hepatic enzymes, low albumin, and hypoxia (Table 1). A noncontrasted head CT was normal, and CSF exhibited 4 WBC/μL (all lymphocytes), 20 RBC/μL, with normal glucose and protein. No organisms were seen on Gram stain of the CSF, and CSF culture did not grow. An anteroposterior chest X-ray showed a widened mediastinum, bilateral hilar masses, right pleural effusion, and bilateral perihilar air space disease. A noncontrasted chest CT scan showed diffuse mediastinal edema; bilateral pleural effusions; bibasilar air space disease; and marked paratracheal, subcarinal, hilar, and azygo-esophageal recess adenopathy. Admission blood cultures grew *B. anthracis* within 15 hours. Ciprofloxacin, rifampin, and clindamycin were begun. On October 22, signs of worsening respiratory distress developed, and on October 23, the patient underwent therapeutic thoracentesis, which yielded bloody pleural fluid, after which his condition improved. He also received systemic corticosteroids for bronchospasm. He required a second thoracentesis and was discharged from the hospital on November 9.

### Case 5

On October 16, a 55-year-old African-American man, a District of Columbia US Postal Service employee, became ill. He had fever, intermittent diaphoresis, myalgias, and cough productive of green sputum but no shortness of breath, chest discomfort, gastrointestinal symptoms, or headache. When he visited his primary-care provider on October 18, he had temperature of 38.9°C, normal heart rate and blood pressure, and respiratory rate of 24/min. He had no other notable examination findings, but WBC count was slightly elevated (Table 1). His past medical history included diabetes mellitus and sarcoidosis. He did not smoke. No chest X-ray was performed. He was sent home with a diagnosis of a viral syndrome; no antibiotics were prescribed. On October 21, he arrived at the emergency department with worsening symptoms, including chest tightness, fatigue, chills, myalgias, nausea, vomiting, and shortness of breath. Temperature was 38.9°C, pulse 93 to 150/min and irregular, respiratory rate 20/min, and blood pressure 119/73 mm Hg. Signs of respiratory distress were observed. Examination findings included rales at the right base with diffuse wheezing and tachycardia. WBC count was 18,800/$mm^3$ with a differential of 73% segmented neutrophils, 6% bands, 11% lymphocytes, and 8% monocytes. Hematocrit was 55%, and platelets were 141,000/$mm^3$. Sodium was 130 mmol/L, potassium 5.3 mmol/L, chloride 99 mmol/L, and bicarbonate 14 mmol/L, with an anion gap of 17. Creatinine was 1.6 mg/dL, and glucose was 425 mg/dL. Aspartate aminotransferase (AST) was 76 IU/L and alanine aminotransferase (ALT) was 77 IU/L. Coagulation studies were normal. Arterial pH was 7.42, $PaCO_2$ 25 mm Hg, $PaO_2$ 66 mm Hg, and $O_2$ saturation 93% on 2 L of $O_2$/min by nasal cannula. A chest X-ray showed right hilar and peritracheal soft tissue fullness with right middle and lower lobe infiltrates compatible with pneumonia and right pleural effusion. An electrocardiogram showed atrial fibrillation. The patient was intubated, ventilated, and administered levofloxacin, diltiazem, and insulin. Later on the day of admission, the patient became hemodynamically unstable, had cardiac arrest, and died. Blood cultures grew *B. anthracis*. Autopsy findings included hemorrhagic mediastinal lymphadenitis, and immunohistochemical staining showed evidence of disseminated *B. anthracis.*

### Case 6

On October 16, a 47-year-old African-American man, a US Postal Service employee who worked at the same District of Columbia mail distribution center associated with cases 3, 4, and 5, had mild nonproductive cough, nausea, vomiting, and stomach cramps. On October 20, the patient had a syncopal episode at church but did not seek medical attention. Early in the morning of October 21, he arrived at an emergency department complaining of vomiting and profuse sweating. His past medical history included asthma and renal calculi. Therapy for asthma had not recently included corticosteroids. He was afebrile and had orthostatic hypotension. WBC count was slightly elevated, but he had normal serum chemistries and coagulation values. Serum glutamic pyruvic transaminase (SGPT) was slightly elevated (Table 1). A chest X-ray was initially read as normal, but later review noted an ill-defined area of increased density due to infiltrate or mass in the right suprahilar region. The patient was discharged after receiving intravenous hydration. On the morning of October 22, he visited the emergency department again, reporting myalgias, chills, dyspnea, continued vomiting, and another syncopal episode. His temperature was 35.6°C, blood pressure 76/48 mm Hg, heart rate 152/min, and respiratory rate 32/min. He was ill-appearing with mottled skin that was cool to the touch, and he was in respi-

ratory distress. He had bilateral wheezing, tachycardia, and mildly distended abdomen with absent bowel sounds. WBC count was 31,200/$mm^3$ with a differential of 78% segmented neutrophils, 2% bands, 14% lymphocytes and 3% monocytes. Sodium was 148 mmol/L, bicarbonate 18 mmol/L, anion gap 21, and creatinine 2.8 mg/dL. Serum glutamic oxalacetic transaminase (SGOT) was 47 IU/L, SGPT 33 IU/L, and alkaline phosphatase 197 IU/L. Prothrombin time was 13.3 seconds and partial thromboplastin time was 40 seconds. Penicillin, ceftriaxone, rifampin, and levofloxacin were begun. Respiratory distress developed, which required endotracheal intubation and mechanical ventilation. Soon thereafter signs consistent with peritonitis were observed. Arterial pH was 7.13, $PaCO_2$ 37 mm Hg, $PaO_2$ 106 mm Hg, and oxygen saturation 95% after intubation on 100% $FiO_2$. A chest X-ray showed bilateral lung infiltrates concentrated within perihilar and infrahilar regions without pleural effusions. Chest and abdominal CT scans with intravenous contrast noted large bilateral pleural effusions, perihilar and suprahilar infiltrates, mediastinal edema, pneumomediastinum, ascites, air in the portal venous system, mesenteric edema, diffuse small bowel edema, and small collections of intramural air involving several parts of the jejunum. CT of the head was normal. The patient died within 6 hours of admission. Gram-positive bacilli were visible on the buffy coat blood smear, and blood cultures grew *B. anthracis* within 18 hours. Postmortem findings included prominent hemorrhagic mediastinal lymphadenitis and evidence of systemic *B. anthracis* infection by histopathologic and immunohistochemical tests.

### Case 7

On the evening of October 22, a 59-year-old Caucasian man, contract employee at a U.S. State Department mail sorting facility that received mail from the District of Columbia postal facility associated with cases 3, 4, 5, and 6, became ill. He had drenching sweats, followed over the next 2 days by fatigue, severe myalgias, subjective fever, chills, headache, nausea, vomiting, abdominal pain, cough with scant white sputum, and substernal chest pain. He had no dyspnea or diarrhea. When he arrived at a local emergency room on October 24, temperature was 38.2°C, heart rate 116/min, and respiratory rate and blood pressure were normal. A complete blood count was normal, and serum electrolytes showed hyponatremia and hypokalemia (Table 1). His past medical history was unremarkable, and he did not smoke. A chest X-ray was initially reported as normal. The patient was thought to have a viral syndrome and was discharged, but blood cultures were obtained and ciprofloxacin was prescribed. He took one dose that night, but vomiting, fatigue, and headache worsened. He also reported transient distortion in his left visual field, and his wife reported that he was intermittently confused. Blood cultures grew gram-positive bacilli after 17 hours of incubation; therefore, on October 25, he was called back to the hospital for admission. The blood isolate was subsequently identified as *B. anthracis*. At admission, his vital signs were as follows: temperature 38.2°C, heart rate 108/min, respiratory rate 20/min, blood pressure 121/60 mm Hg, and oxygen saturation 94% on room air. He appeared ill and had decreased breath sounds at the right base. The rest of the examination was unremarkable. Laboratory studies on admission included WBC count 9,500/$mm^3$ with 81% segmented neutrophils, 9% lymphocytes, and 9% monocytes, hematocrit 48.1%, platelet count 196,000/$mm^3$, normal electrolytes and creatinine, SGOT 85 IU/L, SGPT 64 IU/L, alkaline phosphatase 141 IU/L, bilirubin 1.6 mg/dL, and albumin 3.0 mg/dL. On review, the initial chest X-ray showed mediastinal widening (Figure 5), and chest CT on the day of admission showed mediastinal adenopathy with evidence of hemorrhage, normal lung parenchyma, small bilateral pleural effusions, and a suspected small pericardial effusion (Figure 6). Intravenous penicillin and rifampin were added to the ciprofloxacin. His temperature rose to 39° C. Subsquently, vancomycin was added and penicillin was discontinued. On October 26, gastrointestinal bleeding developed, which required blood transfusion, endoscopic injection, and cautery of gastric and duodenal ulcers. On October 27, atrial fibrillation with variable ventricular response developed. On October 28, fever reached a maximum of 39.4°C and then decreased to 38.3° C. On October 30, WBC peaked at 31,300/$mm^3$. On October 31, enlargement of

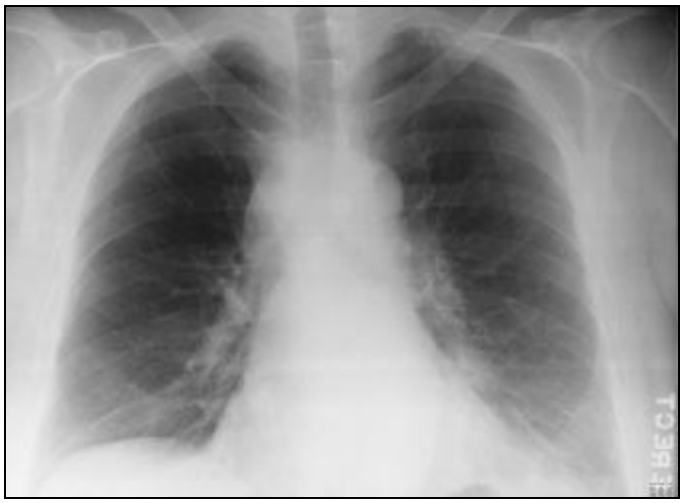

Figure 5. Chest X-ray (Case 7) showing mediastinal widening and a small left pleural effusion.

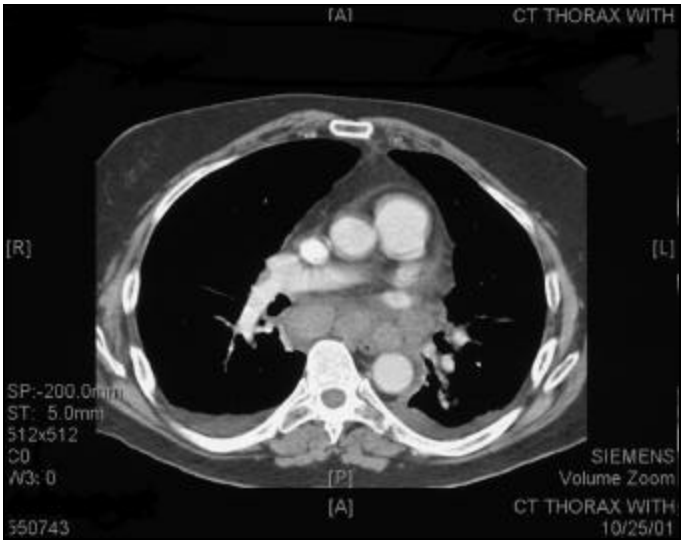


Figure 6. Computed tomography of chest (Case 7) showing mediastinal adenopathy and small bilateral pleural effusions.

the right pleural effusion required thoracentesis and removal of 900 cc of serosanguinous fluid (Table 1). The patient was discharged from the hospital on November 9.

### Case 8

On October 14, a 56-year-old African-American woman who worked as a mail sorter in the Hamilton, New Jersey, US Postal Service facility, became ill with vomiting and diarrhea, followed the next day by subjective fever and chills unrelieved by aspirin. The vomiting and diarrhea improved, but over the next 2 days she had fevers to 38.4 °C with shaking chills, headache, and fatigue. A nonproductive cough developed, along with mild shortness of breath, and anterior chest pain on inspiration. She had no sore throat or rhinorrhea. On October 19, because of persistent fever and worsening chest pain, she went to a local emergency room. Findings at that time included temperature 38.4°C, heart rate 120/min, blood pressure 159/95 mm Hg, and respiratory rate 18/min. She appeared ill with increased respiratory effort, had decreased breath sounds at both bases, and had a 0.5- to 1.0-cm healing scab on the anterior neck. Initial WBC was normal except for elevation in neutrophil band forms, and hematocrit and platelets were normal. Serum electrolytes, creatinine, and coagulation values were unremarkable, except for hyponatremia. Hepatic enzymes were elevated, and she was hypoxic (Table 1). Her past medical history included a transient ischemic attack. She did not smoke. A chest X-ray showed bibasilar infiltrates and a small right pleural effusion but no mediastinal widening. Initial differential diagnosis included atypical pneumonia versus inhalational anthrax, and the patient was begun on levofloxacin; rifampin was added the next day. Two days later, the fever persisted, dyspnea worsened, and large bilateral pleural effusions developed. Antibiotics were changed to ciprofloxacin, rifampin, and vancomycin. Right-sided thoracenteses were performed on October 21 and 22, and 750 cc and 650 cc of hemorrhagic fluid were removed, respectively. On October 23, a right chest tube was placed. A chest CT on October 22 showed mediastinal and cervical lymphadenopathy, bibasilar infiltrates, and large left pleural effusion. On October 25, the enlarging left-sided effusion required chest tube placement. On October 26, the fever resolved, transaminases became normal, and the dyspnea gradually improved. On October 30 and November 1, the chest tubes were removed. On November 5, the patient was discharged from the hospital. Blood for *B. anthracis* DNA by PCR was positive, as were immunohistochemistry studies for *B. anthracis* cell-wall and capsule antigens from pleural fluid cytology preparations.

### Case 9

On October 15, a 43-year-old South Asian woman, also a mail sorter at the US Postal Service facility in Hamilton but at different mail sorting machines from those used by the patient in Case 8, became ill. She had intermittent fevers with chills, dry cough with chest discomfort and shortness of breath, myalgias, and fatigue. She also had nausea and vomiting but no abdominal pain or diarrhea. She complained of "head stuffiness" but no rhinorrhea or sore throat. She also had headache and was reported to be mildly confused. On October 16, when she visited her primary-care physician, she had a temperature of 38°C and was started on levofloxacin for bronchitis. Her past medical history was unremarkable, and she did not smoke. On October 18, she went to a local emergency room because of persistent symptoms.Vital signs were as follows: temperature 38.4°C, heart rate 120/min, respiratory rate 16/min, and blood pressure 141/85mm Hg, with oxygen saturation 92% on room air and 97% on 4 L of oxygen by nasal cannula. She appeared ill and had decreased breath sounds with egophony at the right base. WBC showed increase in neutrophil band forms; hematocrit and platelet count were normal. She had hyponatremia, but electrolytes, renal function, and coagulation values were otherwise normal. Hepatic enzymes were elevated (Table 1). Blood PCR for *B. anthracis* DNA obtained 2 days after initiation of antibiotics was negative. A chest X-ray showed right hilar opacity consistent with consolidation or mass, moderate right and minimal left pleural effusions. Antibiotics were changed to azithromycin and ciprofloxacin. Ciprofloxacin was discontinued 24 hours later. On October 19, a chest CT showed increased soft tissue in the mediastinum (thought to represent adenopathy), right hilar consolidation with possible underlying mass, and large right pleural effusion. Clindamycin and ceftriaxone were added, and thoracentesis was performed with removal of 500 cc of serosanguinous fluid (Table 1). Pleural fluid cytology preparation was positive for *B. anthracis* cell-wall and capsule antigens by immunohistochemical staining. On October 21, repeat thoracentesis was required, and 800 cc of fluid was removed. On October 22, bronchoscopy found edematous, erythematous mucosa. A transbronchial biopsy showed *B. anthracis* cell-wall and capsule antigens by immunohistochemical staining. Cultures of endobronchial samples, pleural fluid, and a nasal swab were all negative for *B. anthracis*, and no other pathogens were identified. On October 23, the fever resolved, other symptoms began to improve, treatment was changed to doxycycline, and the patient was discharged from the hospital on doxycycline on October 26.

### Case 10

On October 25, a 61-year-old Asian woman who worked in the supply room of a New York City Hospital had onset of malaise and myalgias. Over the next 2 days, she also had fatigue, chills, chest pain, progressively worsening dyspnea, and cough productive of sputum, which was later blood-tinged; she denied fever. On October 28, 2001, when she went to a local hospital (other than her workplace), she was noted to be febrile in the emergency room and normotensive, but her heart rate was 110/min and respiratory rate 38/min, with room-air oxygen saturation of 92% by pulse oximetry. She was awake, alert, and completely oriented. She had prominent jugular venous distension at 60 degrees. She had a history of hypertension. She did not smoke. Chest examination showed rales heard to the apices bilaterally. Abdominal and cardiovascular examinations were normal except for tachycardia, and she had no peripheral edema. WBC count was slightly elevated; hematocrit and platelets were normal. Serum chemistries and coagulation studies were normal except for hyponatremia, elevated hepatic enzymes, and hypoalbuminemia. The serum lactate dehydrogenase level was 1,370 IU/L. The patient required oxygen delivery by a nonrebreather mask to maintain adequate oxygenation (Table 1). Chest X-ray was initially interpreted as showing

pulmonary venous congestion with bilateral pleural effusions. Therapy for congestive heart failure was initiated, but an echocardiogram in the emergency department showed normal ejection fraction, no substantial wall motion abnormalities, and a small pericardial effusion. Therapy was changed to levofloxacin for atypical pneumonia. The patient's respiratory status worsened, and she was intubated. A CT scan of the chest showed mediastinal lymphadenopathy, massive mediastinal bleeding, thickened bronchial mucosa, and bilateral pleural effusions. Rifampin, gentamicin and subsequently nafcillin were added to her antimicrobial regimen. Early on October 29, 2001, bilateral chest tubes were placed, and 2.5 liters of serosanguinous fluid was drained from the right side and 1.0 liter from the left (Table 1). On the same day, bronchoscopy showed hemorrhagic mucosa throughout the entire tracheobronchial tree, friable and collapsible airways, and purulent secretions in multiple segments bilaterally. On October 30, the antimicrobial regimen was changed to ciprofloxacin, rifampin, clindamycin, and ceftazidime. Serial echocardiograms documented enlarging pericardial effusion, ultimately with tamponade. The patient's clinical condition progressively worsened, and she died on October 31, after attempted pericardiocentesis. Cultures of blood (after 20 hours) and pleural fluid grew *B. anthracis*. Autopsy findings included hemorrhagic mediastinitis; immunohistochemical stains confirmed the presence of *B. anthracis* in multiple organs.

## Clinical Summary

The median age of patients with inhalational anthrax in this series was 56 years (range 43 to 73 years), 70% were male and, except for the patient in Case 10, all were known to or believed to have processed, handled, or received letters containing *B. anthracis* spores (postal workers [n=6], mail handlers or sorters [n=2], journalist [n=1]). The source of exposure to *B. anthracis* spores in Case 10, a hospital supply room worker, remains undefined. Four patients had underlying cardiovascular or cerebrovascular disease, one had a remote history of treated sarcoidosis and diet-controlled diabetes mellitus, and one had a history of asthma. None of the patients was a current or recent smoker.

The median incubation period from the time of exposure to onset of symptoms when known (n=6) was 4 days (range 4 to 6 days). Two of the patients (in Cases 8 and 9) are presumed to have been exposed on October 9, when a letter later known to be contaminated with spores of *B. anthracis* was processed in the postal facility where they worked. The same letter was routed through a second postal facility and processed on October 12, the presumed day of exposure for four additional cases of inhalational anthrax (Cases 3, 4, 5, and 6).

Patients sought care a median of 3.5 days (range 1 to 7 days) after onset of symptoms. Eight of 10 patients were in the initial phase of illness when they first sought care. Of these eight, six received antibiotics with activity against *B. anthracis* on the same day, and all six survived. Four patients, including one with meningitis, were exhibiting fulminant signs of illness when they first received antibiotics with activity against *B. anthracis*, and all four died. Table 2 summarizes the symptoms of the 10 patients with bioterrorism-related anthrax. Table 3 lists major initial clinical, laboratory and radiologic findings. The median initial WBC count was 9,800/$mm^3$ (range 7,500 to 13,300/$mm^3$). However, the median peak WBC count after presentation and during the course of the illness was 26,400/mm3 (range 11,900 to 49,600/$mm^3$). The chest X-ray was abnormal in all patients, but only seven had mediastinal changes.

Pleural effusions were present in all 10 patients and often became large during hospitalization in those who survived. Seven patients required drainage of pleural fluid, three of these with chest tubes; pleural fluid was consistently hemorrhagic with relatively few leukocytes. Pulmonary infiltrates, which were seen in seven patients, involved the right and left lower lobes (one patient), left upper and lower lobes (one), right upper lobe (two), right lower lobe (one), right middle and right lower lobes (one), and bilateral

Table 2. Symptoms for 10 patients with bioterrorism-related inhalational anthrax, October-November 2001

| Symptoms | n=10 |
|---|---|
| Fever, chills | 10 |
| Fatigue, malaise, lethargy | 10 |
| Cough (minimally or nonproductive) | 9 |
| Nausea or vomiting | 9 |
| Dyspnea | 8 |
| Sweats, often drenching | 7 |
| Chest discomfort or pleuritic pain | 7 |
| Myalgias | 6 |
| Headache | 5 |
| Confusion | 4 |
| Abdominal pain | 3 |
| Sore throat | 2 |
| Rhinorrhea | 1 |

Table 3. Initial clinical findings in 10 patients with bioterrorism-related inhalational anthrax, October - November 2001

| | |
|---|---|
| Physical findings | |
| Fever (>37.8°C) | 7/10 |
| Tachycardia (heart rate >100/min) | 8/10 |
| Hypotension (systolic blood pressure <110 mm Hg) | 1/10 |
| Laboratory results | |
| White blood cell count (median, range) | 9.8 x $10^3$/$mm^3$ |
| Differential – neutrophilia (>70%) | 7/10 |
| Neutrophil band forms (>5%) | 4/5 |
| Elevated transaminases[a] (SGOT or SGPT > 40) | 9/10 |
| Hypoxemia (Alveolar-arterial oxygen gradient >30mm Hg on room air $O_2$ saturation <94%) | 6/10 |
| Metabolic acidosis | 2/10 |
| Elevated creatinine (>1.5 mg/dL) | 1/10 |
| Chest X-ray findings | |
| Any abnormality | 10/10 |
| Mediastinal widening | 7/10 |
| Infiltrates/consolidation | 7/10 |
| Pleural effusion | 8/10 |
| Chest computed tomography findings | |
| Any abnormality | 8/8 |
| Mediastinal lymphadenopathy, widening | 7/8 |
| Pleural effusion | 8/8 |
| Infiltrates, consolidation | 6/8 |

[a]SGOT = serum glutamic oxalacetic transaminase; SGPT = serum glutamic pyruvic transaminase

perihilar areas (one). Eight patients had a chest CT performed; mediastinal lymphadenopathy was present in all but one patient.

Blood cultures were obtained before antibiotic therapy was initiated in seven patients, and *B. anthracis* was isolated in all seven. Blood cultures grew *B. anthracis* at a median of 18 hours (range 12 to 24 hours). Three patients had the first set of blood cultures obtained after initiation of antibiotic therapy; these cultures revealed no growth. One patient with blood cultures that grew *B. anthracis* had blood cultures repeated within 24 hours after initiating antibiotics with activity against *B. anthracis*, and the repeat cultures did not grow.

*B. anthracis*-specific immunohistochemical tests performed on pleural fluid cytology preparations or transbronchial biopsy specimens were positive in every case in which these tissues were available (three nonfatal cases) (Figure 7A). Three patients had atrial arrythmias (supraventricular tachycardia in Case 2 and atrial fibrillation in Cases 5 and 7), and three patients had pericardial effusion on CT scan. In one patient, pericardial tamponade was suspected. Six of 10 patients with inhalational anthrax have survived to date (death rate 40%). Five have been discharged from the hospital, and one is recovering in the hospital. Figure 8 shows the timeline from exposure (when known) to current status for these 10 patients. Autopsy findings in all four patients who died showed hemorrhagic mediastinal lymphadenitis and evidence of disseminated *B. anthracis* infection (Figure7B-D)

## Discussion

Before this outbreak of bioterrorism-related anthrax, only 18 cases of inhalational anthrax had been reported in the United States in the 20th century (1,4,9-21). The most recent case was in 1976 (4). Most cases were related to exposure to animal products, primarily in textile mills processing goat hair, goat skins, or wool (1). Clinical characteristics in the 10 cases due to bioterrorism described here share similarities with previously reported cases but have important differences.

Inhalational anthrax has been described as a biphasic clinical illness characterized by a 1-to 4-day initial phase of malaise, fatigue, fever, myalgias, and nonproductive cough, followed by a fulminant phase of respiratory distress, cyanosis, and diaphoresis (1). Death follows the onset of the fulminant phase in 1 to 2 days (2,22). The symptoms of the initial phase of inhalational anthrax in the 10 cases caused by bioterrorism (Table 1) were similar to those of the 18 occupationally related cases previously described (1); however, profound, often drenching sweating, which was a prominent feature in the current cases, was not emphasized in earlier reports. Previous case reports have noted a brief period of improvement between the initial and fulminant phases (1), but this phenomenon was not observed in the current cases. Nausea and vomiting were also frequent symptoms of the initial phase in the current cases, suggesting early involvement of the gastrointestinal tract. Gastrointestinal lesions were observed in 39 of 42 total cases of fatal inhalational anthrax associated with the Sverdlovsk outbreak (23). Most lesions in these fatal cases appeared to represent hematogenous spread of *B. anthracis* to the submucosa of the gastrointestinal tract and did not involve Peyer's patches or, in most cases, mesenteric lymph nodes. The nondistinctive nature of the initial phase of inhalational anthrax presents a diagnostic challenge. The chest X-ray appeared to be a sensitive indicator of disease in patients with bioterrorism-associated inhalational anthrax, as none of the 10 patients had an initially normal chest X-ray. Multiple abnormalities were noted on initial chest X-ray, including mediastinal widening, paratracheal fullness, hilar fullness, pleural effusions,and parynchemal infiltrates. Two patients had chest X-rays that were interpreted initially as normal, but abnormalities (mediastinal widening in one case and perihilar mass versus

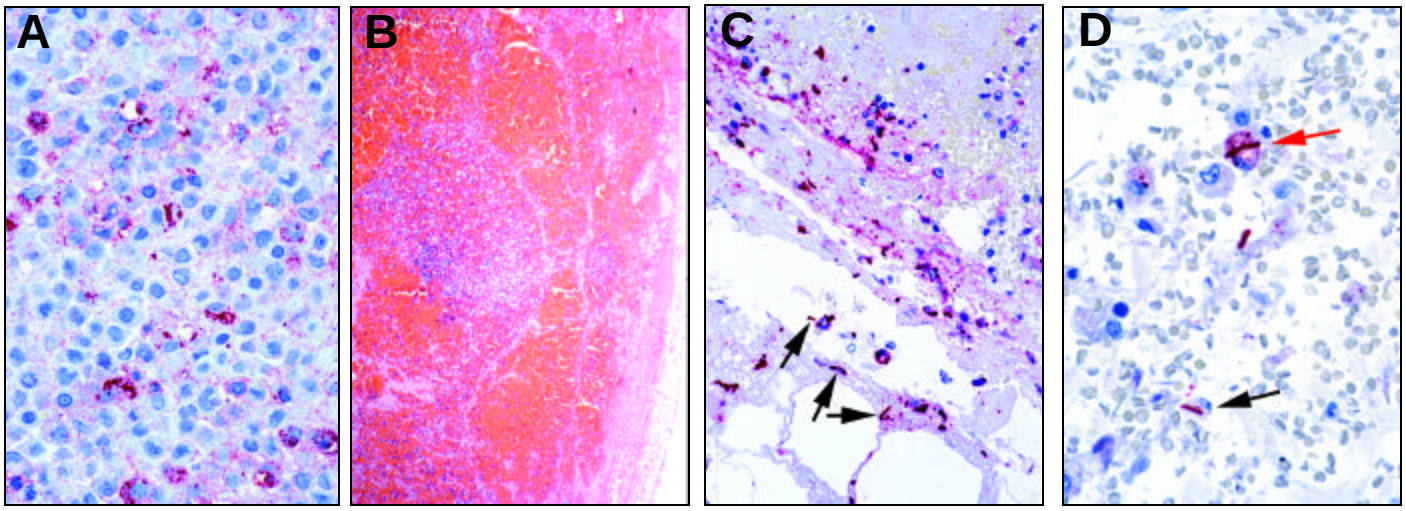


Figure 7(A). Pleural fluid cell block from a nonfatal case showing abundant *Bacillus anthracis* granular antigen staining inside mononuclear inflammatory cells. (Immunohistochemical assay with a mouse monoclonal anti-*B. anthracis* capsule antibody and detection with alkaline phosphatase and naphthol fast red, original magnification 158X). (B) Mediastinal lymph node from a fatal case of anthrax showing extensive capsular and sinusoidal hemorrhage. (Hematoxylin and eosin, original magnification 25X). (C) Lymph node from same case shown in B, showing abundant *B. anthracis* granular antigen staining inside mononuclear inflammatory cells and bacilli (arrows) in the subcapsular hemorrhagic area. (Immunohistochemical assay with a mouse monoclonal anti-*B. anthracis* cell wall antibody and detection with alkaline phosphatase and naphthol fast red, original magnification 100X). (D) Lung tissue from a fatal case showing *B. anthracis* granular antigen staining inside a perihilar macrophage (red arrow) and intra- and extracellular bacilli (black arrow). (Immunohistochemical assay with a mouse monoclonal anti-*B. anthracis* cell wall antibody and detection with alkaline phosphatase and naphthol fast red, original magnification 100X)

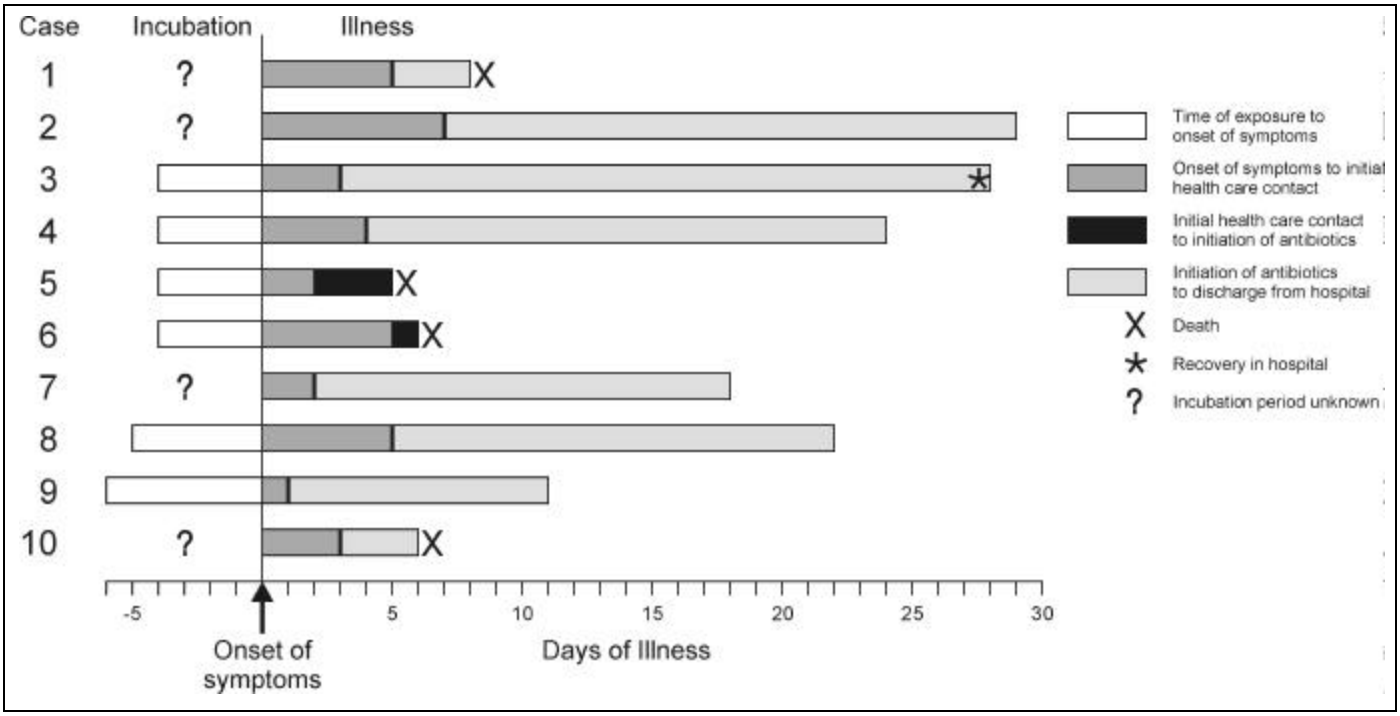


Figure 8. Timeline of 10 cases of inhalational anthrax in relation to onset of symptoms, October through November 2001.

infiltrate in the other) were detected when the X-rays were reviewed by a radiologist. Pulmonary infiltrates or effusion were initially seen in two cases without evidence of mediastinal widening. Chest CT was helpful in further characterizing abnormalities in the lungs and mediastinum and was more sensitive than chest X-ray in revealing mediastinal lymphadenopathy.

The total WBC count was usually normal or only slightly elevated at the time of initial visit to a health-care provider for patients who sought medical care in the initial phase. However, an elevation in the proportion of neutrophils or band forms was frequently noted and was an early diagnostic clue. During the course of illness, WBC counts increased, sometimes markedly, in most patients.

Blood cultures grew *B. anthracis* even in the initial phase of the illness in all patients who had not received prior antibiotic therapy. Animal models suggest that primates with inhalational anthrax become bacteremic early in the course of the illness before the fulminant clinical phase develops (24). In contrast, blood cultures rapidly became sterile after initiation of antibiotic therapy, suggesting that prior antibiotic treatment may substantially decrease the sensitivity of blood cultures as a diagnostic test.

The diagnosis of anthrax was established in three patients without growth of *B. anthracis* from clinical specimens. In all three of these cases, proper cultures were obtained only after initiation of antibiotic therapy. The diagnosis in these patients was established by a history of exposure or occupational and environmental risk with a clinically compatible syndrome, by the identification of *B. anthracis* in pleural fluid, pleural biopsy, or transbronchial biopsy specimens by immunohistochemical staining with *B. anthracis*-specific cell wall and capsular antibodies, or by identifying *B. anthracis* DNA by PCR on pleural fluid or blood. Serologic data from ELISA available for one patient with inhalational anthrax also demonstrated a >4-fold increase in levels of serum antibody (IgG) to the PA component of anthrax toxins.

The survival of patients with inhalational anthrax in this series (60%) is higher than previously reported (<15%) (1,3). All patients received combination antimicrobial therapy with more than one agent active against *B. anthracis*. The apparent improvement in survival compared with previous cases suggest that the antibiotic combinations used in these patients may have therapeutic advantage over previous regimens. Limited data on treatment of the survivors suggests that early treatment with a fluoroquinolone and at least one other active drug (7) may improve survival. Other antimicrobial susceptibilities of the isolates associated with this outbreak have been published (7). *B. anthracis* isolates produce a cephalosporinase (7) that inhibits the antibacterial activity of cephalosporins such as ceftriaxone, and cephalosporins should not be used for treatment. Other explanations for the improved survival rate include earlier recognition and initiation of treatment, better supportive care, differences in the pathogenesis of bioterrorism-related anthrax, differences in susceptibility of the hosts, or a combination of the above.

Pleural effusions were a remarkably consistent clinical feature of inhalational anthrax in this series, occurring in all patients. The pleural effusions were often small on presentation, but in the surviving patients effusions were characterized by progressive enlargement and persistence. Drainage of the pleural cavity was required in seven patients. The characteristics of the pleural fluid in all patients were similar: hemorrhagic, with a high protein concentration and relatively few WBCs. Immunohistochemistry demonstrated large quantities of *B. anthracis* capsule and cell-wall antigens in pleural tissue or pleural fluid cell blocks.

Case 2 was remarkable in that X-ray findings were dominated by large and progressive pulmonary infiltrates, not by mediastinal widening. In fact, no mediastinal adenopathy was noted on chest CT. Transbronchial biopsies of the patient in Case 2 showed *B. anthracis*-specific capsular and cell-wall antigens in the lung parenchyma. Previous reports have noted bronchopneumonia and pulmonary hemorrhagic infarcts in patients with inhalational anthrax. Abramova et al. (23) described focal hemorrhage and necrotizing anthrax pneumonia in 11 of 42 patients who died with inhalational anthrax. Characteristics of the spore-containing aerosol or individual host factors may influence pathogenesis, so pulmonary infiltrates may be a more prominent manifestation in some patients with bioterrorism-related inhalational anthrax. Three patients had supraventricular arrythmias, and three had pericardial effusion on chest CT, one of which may have progressed to tamponade. Pericardial effusions may reflect toxin-related local edema or inflammation, hemorrhagic necrosis and infarct extending into the pericardium from the mediastinum, or hematogenous spread of *B. anthracis.*

Anthrax meningitis is a complication of inhalational anthrax and is characteristically hemorrhagic. Pathologic findings are a hemorrhagic leptomeningitis with edema and inflammatory infiltrates (2,23). Fifty-five percent of patients at Sverdlovsk who died of inhalational anthrax had evidence of meningeal involvement at autopsy. Cerebrospinal fluid was examined in only two patients in our series, and meningitis was documented in one. Three other patients had a history of intermittent confusion, but no meningeal signs were observed, and lumbar punctures were not performed. No predominant underlying diseases or conditions were noted in the patients with bioterrorism-related inhalational anthrax. One patient (Case 5) had a remote history of sarcoidosis but had been free of clinical illness or treatment associated with this diagnosis for the past 25 years. One other case of inhalational anthrax associated with underlying sarcoidosis has been reported (12). The patient in Case 5 also had diabetes mellitus, a condition present in one previously reported case (12). Smoking did not appear to be a risk factor for inhalational anthrax.

In summary, we describe the clinical presentation of the first 10 cases of bioterrorism-related inhalational anthrax in the United States. The clinical presentation in these patients was variable and often resembled a viral respiratory illness, but the interpretation of the initial symptoms in the context of a possible exposure to *B. anthracis* often led to an early diagnosis. In contrast to previous reports indicating a death rate >85% (1,3), our series suggests that survival may be markedly improved by combination antimicrobial therapy begun during the initial phase of the illness and by aggressive supportive care (e.g., drainage of pleural effusions). Newer methods of detection such as polymerase chain reaction, immunohistochemistry, and sensitive serologic tests are important adjunctive diagnostic modalities that aid in the diagnosis of *B. anthracis* infections. Further studies are needed to better define optimal antimicrobial regimens, explore the role of adjunctive therapies (e.g., immunoglobulin antitoxin, corticosteroids, and other toxin inhibitors), and better understand the pathogenesis of inhalational anthrax associated with intentional release.

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Dr. Jernigan is a medical epidemiologist with the National Center for Infectious Diseases, Centers for Disease Control and Prevention, and assistant professor of medicine with Emory University School of Medicine. He has been extensively involved in the clinical evaluation of the anthrax cases associated with this outbreak.

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# Advanced Age a Risk Factor for Illness Temporally Associated with Yellow Fever Vaccination

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In 1998, the Centers for Disease Control and Prevention was notified of severe illnesses and one death, temporally associated with yellow fever (YF) vaccination, in two elderly U.S. residents. Because the cases were unusual and adverse events following YF vaccination had not been studied, we estimated age-related reporting rates for systemic illness following YF vaccination. We found that the rate of reported adverse events among elderly vaccinees was higher than among vaccinees 25 to 44 years of age. We also found two additional deaths among elderly YF vaccinees. These data signal a potential problem but are not sufficient to reliably estimate incidence rates or to understand potential underlying mechanisms; therefore, enhanced surveillance is needed. YF remains an important cause of severe illness and death, and travel to disease-endemic regions is increasing. For elderly travelers, the risk for severe illness and death due to YF infection should be balanced against the risk for systemic illness due to YF vaccine.

In 1998, the Centers for Disease Control and Prevention (CDC) was notified of severe illnesses occurring days after immunization with yellow fever (YF) vaccine in two elderly U.S. residents. Both vaccinees had been in good health, and neither was immunocompromised. One patient died. Because these cases were unusual and the risk for illness following vaccination with YF vaccine in the elderly had not been studied, we estimated age-related reporting rates for YF vaccine associated systemic illness.

YF is an acute febrile illness caused by a mosquito-borne flavivirus (1). Clinical presentation ranges from a mild, febrile illness to a life-threatening infection involving hepatic failure, renal dysfunction, myocardial injury, and a bleeding diathesis. YF is endemic in much of tropical South America and sub-Saharan Africa (2).

Two live, attenuated YF vaccines were developed in the early 1930s, the French neurotropic vaccine (FNV) and the 17D vaccine (1,3-5). Production of FNV was halted in 1982 because its neurotropism had resulted in cases of encephalitis, primarily among children (1,6,7). Derivatives of the 17D strain are the only YF virus strains currently used for vaccine production. These vaccines are not cloned from a single virus but consist of a heterologous population of virions (8). Human trials with the 17D YF vaccine in the 1930s found low rates of adverse events and protective levels of YF viral neutralizing antibodies in more than 95% of vaccinees (3,5). More recent studies have shown that protective antibodies may last 30 to 35 years (9).

Early field trials and experiments with the 17D virus demonstrated that virulence varied with the passage level. Some substrains were overattenuated and led to low rates of seroconversion, while others were associated with post-vaccine encephalitis (10,11). A seed lot system, which standardizes vaccine preparation and limits passage of the virus, was recognized as the production standard in 1945 (1,12). The World Health Organization publishes recommended standards. Previous reports of YF adverse events have focused primarily on hypersensitivity or neurologic sequelae. A review of reports submitted to the Vaccine Adverse Event Reporting System (VAERS) in the United States from 1990 to 1997 found a rate of probable anaphylaxis after YF vaccine immunization of 1 per 131,000 vaccine doses distributed

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(13). Since the seed lot system was introduced, 21 cases of post-vaccine encephalitis have been reported worldwide (20 patients recovered, one died) (1,14); 16 (76%) of these cases occurred in children <9 months. Meningoencephalitis has on rare occasions been reported among adults after immunization with the vaccine (15,16), and severe, multisystemic illness has recently been reported in seven YF vaccinees (17-19).

YF vaccine has had a long history of efficacy and presumed safety (1). Nonetheless, a reexamination of its safety profile has been prompted by its increased use in international travelers and by these recent reports of serious adverse events (17-19).

## Methods

Adverse events following vaccination with YF vaccine reported to VAERS were collected and categorized as systemic, nonsystemic, or unrelated and were classified by age group. The number of doses administered by age group (denominators) was estimated from the age distribution of travelers receiving the vaccine at a sample of travel clinics and from the number of doses distributed to civilians in the United States by the vaccine manufacturer. Reporting rates for systemic and nonsystemic adverse events were calculated by dividing the events reported by an estimate of the number of people receiving the vaccine in each age group.

### Source of Cases and Case Classification

VAERS, a passive surveillance system for adverse events, monitors vaccine safety in the United States and is jointly operated by CDC and the U.S. Food and Drug Administration (20). All civilian U.S. VAERS reports from 1990 through 1998 listing YF vaccine were reviewed. Reports of death, hospitalization or disability, a life-threatening illness, or illness requiring an emergency room or doctor visit were analyzed. Reports that did not involve any of these events were considered less serious and were excluded from analysis. Reports were blinded for age and reviewed independently by three physicians. Adverse events were classified as neurologic, multisystemic, uncomplicated neurologic or systemic, nonspecific, hypersensitivity, local reactions, or unrelated (Table 1). If more than one category was appropriate, the most serious category, in terms of reaction to the specific vaccine components, was selected. These categories were defined for the purposes of this study and reflect an interest in examining adverse events that might be related to the vaccine virus rather than those that might be immune responses to other vaccine components. The investigators reached a consensus on the categorization of each report before unblinding the ages.

A systemic adverse event (SyAE) was defined as a multisystemic (excluding anaphylactic) or neurologic reaction. Adverse events categorized as uncomplicated neurologic or systemic, hypersensitivity, or local reaction were defined as other adverse events (OAE). A second analysis used a more stringent definition of SyAE that included only neurologic or multisystemic cases requiring hospitalization or resulting in death (SyAE*).

VAERS reports that did not include the age of the vaccinee, provided another explanation of the adverse event (e.g., local reaction from another vaccination), or indicated inappropriate administration or inadvertent use (e.g., during pregnancy) were excluded. Reports from children <15 years of age and military personnel were excluded because no adequate estimates of the number of persons who received YF vaccine in these groups were available. As a comparison, similar analyses were done on adverse events after hepatitis A (HA) vaccine reported to VAERS during 1994 to 1998 (Table 1).

VAERS solicits reports not only of events known to be causally related to vaccine but also of all events temporally related to vaccination, some of which may be coincidental. Evaluating the causal relationship of an event to a specific vaccine may be also confounded by the routine practice of administering multiple vaccines at a single visit. Furthermore, VAERS has several other methodologic limitations inherent to passive surveillance systems, such as under-, biased-, and incomplete reporting and lack of consistent diagnostic criteria. Thus, VAERS reporting rates are, at best, a crude estimate of event rates. Given these limitations of passive surveillance systems, neither reporting rates nor

Table 1. Categories of vaccine adverse events[a]

| |
|---|
| **Neurologic (SyAE)** |
| • Guillain-Barré syndrome, new onset seizures, encephalitis, myelitis, altered mental status, focal cranial or peripheral neurologic deficits, paresthesias, vertigo, headaches (headaches alone are not sufficient for neurologic diagnosis)[b] |
| • Onset <2 weeks after vaccination |
| • Duration >72 hours |
| **Multisystemic (SyAE)** |
| • Myalgias, arthralgias, rhabdomyolysis, elevated transaminases, respiratory distress, nausea, vomiting, diarrhea, nephropathy, disseminated intravascular coagulation, +/-fever[b] |
| • Onset <2 weeks after vaccination |
| • Duration >72 hours |
| **Neurologic/systemic, uncomplicated (OAE)** |
| • Cases that met the neurologic or systemic criteria but had a full and rapid clinical recovery in <72 hours |
| **Nonspecific events without other focal finding (OAE)** |
| • Dizziness, headache |
| • Nausea, vomiting, or diarrhea alone |
| **Hypersensitivity (OAE)** |
| • Rash, urticaria, +/- fever |
| • Anaphylaxis, angioedema |
| • Onset within 48 hours of vaccination |
| **Local reaction (OAE)** |
| • Localized pain, swelling, erythema, or warmth (at injection site) |
| • Onset within 1 week of vaccination |
| **Unrelated to vaccine (excluded from AE analysis)** |
| • A clear, alternative diagnosis confirmed by laboratory criteria accounts for symptoms and signs; sometimes this is an underlying illness |
| • Another cause implied or stated in the physician's report |
| • For hepatitis A vaccine, onset of adverse event is >6 weeks |

SyAE = systemic adverse event; OAE = other adverse events; AE = adverse event(s).
[a]Listed in order from most to least severe.
[b]Examples, but not limited to these signs, symptoms and conditions.

the number of events reported to VAERS may automatically be considered synonymous with the incidence of adverse events. Elevated VAERS reporting rates may best serve as sentinel signals suggesting hypotheses to test in other more rigorous databases before definitive conclusions can be reached.

### Denominator

The sole manufacturer of YF vaccine in the United States (now known as Aventis Pasteur) provided the annual number of YF vaccine doses purchased by civilian providers from 1995 through 1998. The annual number of doses from 1990 through 1994 was extrapolated from the number of doses in 1995. We assumed that the number of doses increased each year at the same annual rate as occurred from 1995 to 1996. Doses for 1997 and 1998 were not used in the extrapolation because unusual supply and regulatory issues influenced the number of doses provided in these years. Telephone interviews with health-care providers indicated little or no waste of YF vaccine, which for civilian use in the United States is sold predominantly in single-dose vials. Thus, it was assumed that the total number of doses sold was a good estimate of the total number of doses administered.

The manufacturers of HA vaccine (Havrix, SmithKline Beecham, Rixensart, Belgium; and Vaqta, Merck & Co., Inc., West Point, PA) provided the annual number of doses of HA vaccine purchased from 1995 through 1998. We estimated that 10% of HA vaccine was wasted and that 50% of vaccinees received both doses in the series. The total number of doses sold was reduced by these amounts to estimate the total number of doses administered. These doses did not include those used for outbreak control.

Thirteen U.S.-based GeoSentinel clinics, which provide YF vaccine to international travelers, reviewed records of YF and HA vaccine administration. GeoSentinel is an international network of travel and tropical medical clinics established in 1995 as a collaborative effort by CDC and the International Society of Travel Medicine (21). GeoSentinel monitors geographic and temporal infectious disease trends among people crossing international borders; the clinics are chosen to detect sentinel events in travelers seen in clinics before and after travel. Within the United States, these clinics are considered representative of clinics that offer YF vaccine. All YF and HA vaccine recipients during the most recent 12-month period for which complete information was available were categorized by age group (15 to 24 years, 25 to 44 years, 45 to 64 years, 65 to 74 years, and ≥75 years). Data for children <15 years of age and military personnel were excluded because these groups are underrepresented at U.S. GeoSentinel clinics. Other than these exceptions, the age distribution for YF vaccine recipients at GeoSentinel clinics was assumed to represent national YF vaccine use. The age distribution for HA vaccine recipients from these clinics was also assumed to represent national use excluding the same exceptions and outbreak control.

The number of YF vaccine doses administered to each age group was estimated by multiplying the total number of YF vaccine doses per year by the proportionate age group distribution estimated from the GeoSentinel clinics. Age group-specific reporting rates for SyAE per 100,000 doses and reporting rate ratios for SyAE were calculated with a reference group of 25- to 44-year-old vaccine recipients. The 25- to 44-year-old group was chosen because of the previously reported increased risk for adverse events among younger YF vaccine recipients (1). Although the risk is highest for infants <4 months of age, it is unclear at what age the risk reaches a nadir. Confidence intervals were calculated based on standard statistical assumptions for confidence intervals (CI) for ratios of rates, although because of the limitations of passive surveillance systems, these assumptions may not hold.

## Results

From 1990 through 1998, VAERS received 166 reports of YF vaccine adverse events that met the criteria for review (Figure 1). Thirty-five (21%) of these reports were categorized as SyAEs and 36 (22%) as OAEs. Of the 10 VAERS reports for patients >65 years of age, one was categorized as an OAE and the other nine as SyAE. The latter included the two index patients, one additional death, and six patients with various signs and symptoms, including fever, headache, malaise, myalgia, nausea, somnolence, and ataxia. Two of these six patients were hospitalized. Ninety-five (57%)

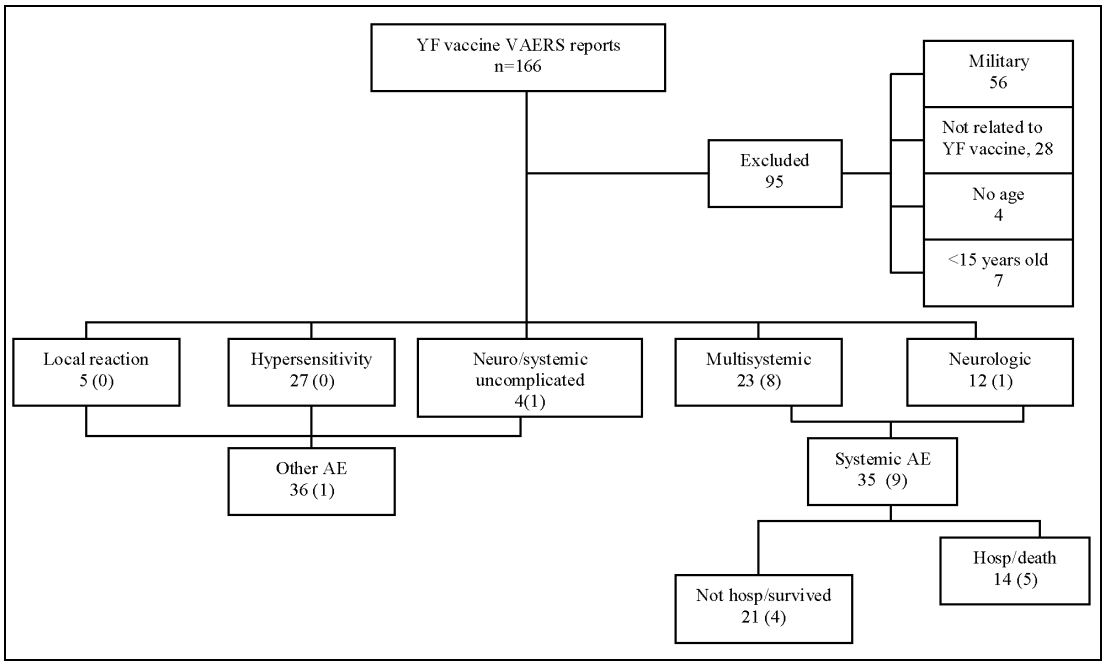


Figure 1. Diagram of yellow fever (YF) vaccine adverse events reports (1990-1998). n = number of reports; O = number of reports in patients >65 years old. VAERS = Vaccine Adverse Event Reporting System.

reports were excluded because they did not include the age of the vaccinee (4 reports), or the vaccine was used in military personnel (56 reports) or a child <15 years old (7 reports), or an alternative cause of the adverse event was reported (28 reports). Seventeen patients were hospitalized, and three patients, ages 63, 67, and 79 years, died. The clinical course for the two index patients and the two additional deaths was characterized by a nonspecific febrile syndrome with fatigue, myalgia, and gastrointestinal symptoms, rapidly progressing to a severe multisystemic illness with dysfunction of liver, kidneys, lungs, central nervous system, as well as thrombocytopenia, and possible disseminated intravascular coagulopathy (18).

From 1990 through 1998, U.S. civilian providers purchased an estimated 1.55 million doses of YF vaccine. The age distribution of YF vaccine recipients was estimated from 5,125 YF vaccine recipients in 13 GeoSentinel clinics. The reference group, ages 25 to 44 years, accounted for 45% of the sample; 285 (5.6%) of the vaccinees were 65 to 74 years of age, and 73 (1.4%) were ≥75 years of age (Figure 2).

The overall reporting rate for a SyAE after YF vaccination was 2.4 per 100,000 doses, and the reporting rate for death was 0.2 per 100,000 doses; for those ≥65 years of age, the overall reporting rate for SyAE was 8.3 per 100,000 doses, and the reporting rate for death was 1.8 per 100,000 doses. During this period, an estimated 108,000 doses were administered to those ≥65 years of age. The reporting rate for a SyAE for the reference age group (25 to 44 years) was 1.6 per 100,000 doses and increased progressively for each older age group to 5.8 per 100,000 doses for vaccinees 65 to 74 years of age and 18.1 per 100,000 doses for those ≥75 years of age (Table 2). Compared with the reference group, the reporting rate ratio for an SyAE for those 65 to 74 years of age was 3.7 (95% CI 1.3-10.7); for those ≥75 years of age, it was 11.6 (95% CI 3.7-36.3). Conversely, the reporting rate for OAE decreased for each older age group.

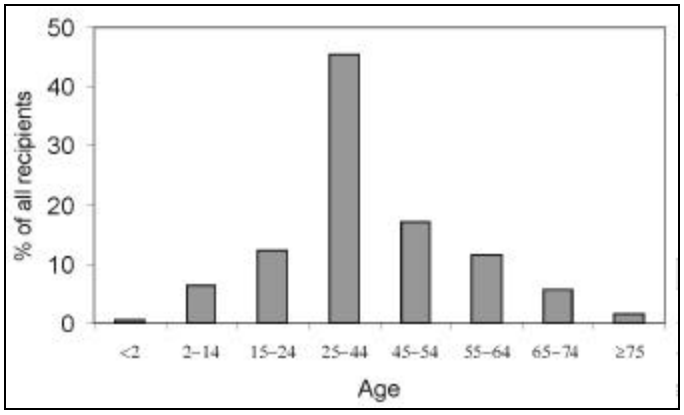


Figure 2. Estimated age range for YF vaccine recipients, n=5,125. Percentage of children ≤15 years of age is underestimated as these groups were excluded from analysis (see text).

When analysis of SyAEs was restricted to vaccinees who died or were hospitalized, the pattern was even stronger (Table 3). Compared with the reference group, the reporting rate ratio for an SyAE* for those 65 to 74 years of age was 12.3 (95% CI 2.0-73.2); for those ≥75 years of age, it was 31.8 (95% CI 4.5-225.9).

Of the 35 patients with SyAEs, 19 (54%) received at least one other vaccine in addition to YF vaccine. When analysis was restricted to those who received only YF vaccine, the reporting rate ratio for a SyAE* for those 65 to 74 years of age was 6.1 (95% CI 1.4-27.3); for those ≥75 years of age it was 23.9 (95% CI 5.3-106.6). Six of the nine patients ≥65 years of age with SyAEs received only YF vaccine, and all five patients ≥65 years of age who were hospitalized or died had received that vaccine alone.

During 1995 to 1998, VAERS received 310 reports of adverse events after immunization with HA vaccine that met the inclusion criteria. From an estimated 3.2 million doses of HA vaccine, 30 patients were hospitalized, and none died. This is double the total number of doses of YF vaccine and almost three times the number of YF vaccine doses given to people ages ≥75 years. The reporting rate for an SyAE after HA vaccine for vaccine recipients 65 to 74 years of age was 6.2 per 100,000. This was higher than the 2.5 per 100,000 reporting rate for the reference group (ages 25 to 44 years); however, there was no consistent increase in the reporting rate for SyAE for each older age group, and recipients ages ≥75 years did not have a different reporting rate from those ages 25 to 44 years (reporting rate ratio =1.9, 95% CI 0.6-6.3) (Table 4) (Figure 3).

## Discussion

Severe illness in two elderly recipients of YF vaccine, one of whom died shortly after immunization, prompted this collaborative study, which examined reporting rates for adverse events among elderly YF vaccine recipients in the United States (18). We found a higher reporting rate for SyAEs among elderly YF vaccine recipients than among YF vaccine recipients ages 25 to 44 years. This increase in reporting rates persisted when adverse events were limited

Table 2. Reporting rates (RR) and reporting rate ratios (RRR) for yellow fever (YF) vaccine adverse events by age, 1990–1998

| Age (years) | No. vaccine doses | No. OAE[a] | OAE reports/ 100,000 doses | RRR (95% CI) | No. SyAE[b] | SyAE reports/ 100,000 doses | RRR (95% CI) |
|---|---|---|---|---|---|---|---|
| 15-24 | 189,991 | 11 | 5.79 | 2.5 (1.2-5.5) | 3 | 1.58 | 1.0 (0.3-3.6) |
| 25-44 | 702,783 | 16 | 2.28 | Reference | 11 | 1.57 | Reference |
| 45-64 | 442,605 | 8 | 1.81 | 0.8 (0.3-1.9) | 12 | 2.71 | 1.7 (0.8-3.9) |
| 65-74 | 86,222 | 1 | 1.16 | 0.5 (0.1-3.8) | 5 | 5.80 | 3.7 (1.3-10.7) |
| ≥75 | 22,085 | 0 | 0 | undefined | 4 | 18.11 | 11.6 (3.7-36) |
| Total | 1,443,686 | 36 | 2.49 | | 35 | 2.42 | |

CI = confidence interval.
[a]OAE: other adverse event (uncomplicated neurologic/systemic, hypersensitivity, or local reaction).
[b]SyAE: systemic adverse event (multisystemic [excluding anaphylactic] or neurologic reaction)

Table 3. Reporting rates (RR) and reporting rate ratios (RRR) for serious systemic yellow fever (YF) vaccine adverse events (SyAE*) by age, 1990–1998

| Age (years) | No. vaccine doses | No. of OAE*[a] | OAE* reports/ 100,000 doses | RRR (95% CI) | No. SyAE*[b] | SyAE* reports/ 100,000 doses | RRR (95% CI) |
|---|---|---|---|---|---|---|---|
| 15-24 | 189,991 | 12 | 6.32 | 1.8 (0.9-3.5) | 2 | 1.05 | 3.7 (0.5-26) |
| 25-44 | 702,783 | 25 | 3.56 | Reference | 2 | 0.29 | Reference |
| 45-64 | 442,605 | 15 | 3.39 | 1.0 (0.5-1.8) | 5 | 1.13 | 4.0 (0.8-20) |
| 65-74 | 86,222 | 3 | 3.48 | 1.0 (0.3-3.2) | 3 | 3.48 | 12.3 (2.0-73) |
| ≥75 | 22,085 | 2 | 9.06 | 2.5 (0.6-10.7) | 2 | 9.06 | 32 (4.5-226) |
| Total | 1,443,686 | 57 | 3.95 | | 14 | 0.97 | |

CI = confidence intervals.
[a]OAE*= other adverse events (i.e., uncomplicated neurologic or systemic event; hypersensitivity; local reaction) OR systemic adverse events not requiring hospitalization or resulting in death.
[b]SyAE* = serious systemic adverse events, including only neurologic or multisystemic adverse events requiring hospitalization or resulting in death; this is distinguished from the term SyAE, which indicates all systemic adverse events (See Table 2).

Table 4. Reporting rates (RR) and reporting rate ratios (RRR) for hepatitis A vaccine adverse events by age, 1995–1998

| Age (years) | No. HA doses | No. OAE[a] | OAE reports/ 100,000 doses | RRR (95% CI) | No. SyAE[b] | SyAE reports/ 100,000 doses | RRR (95% CI) |
|---|---|---|---|---|---|---|---|
| 15-24 | 387,031 | 21 | 5.43 | 1.8(1.1-3.0) | 7 | 1.81 | 0.7 (0.3-1.6) |
| 25-44 | 1,444,895 | 44 | 3.05 | Reference | 36 | 2.49 | Reference |
| 45-64 | 1,096,391 | 23 | 2.10 | 0.7 (0.4-1.1) | 26 | 2.37 | 1.0 (0.6-1.6) |
| 65-74 | 241,453 | 4 | 1.66 | 0.5 (0.2-1.5) | 15 | 6.21 | 2.5(1.4-4.6) |
| ≥75 | 61,760 | 1 | 1.62 | 0.5 (0.1-3.9) | 3 | 4.86 | 1.9 (0.6-6.3) |
| Total | 3,231,530 | 93 | 2.88 | | 87 | 2.69 | |

HA = hepatitis A [vaccine]; CI = confidence intervals.
[a]OAE = other adverse events (uncomplicated neurologic/systemic, hypersensitivity, or local reaction).
[b]SyAE: systemic adverse event (multisystemic [excluding anaphylactic] or neurologic reaction)

to patients who required hospitalization or died and when recipients who also received other vaccines were excluded. Although we did find an elevated rate of reported SyAEs following HA vaccine in the 65- to 74-year-old group, we did not find a similar increase in persons ≥75 years, despite almost three times as many doses sold overall and twice as many adverse events reported to VAERS for all age groups combined. No deaths following HA vaccination were reported.

Our analysis showed that the reporting rate for systemic illness requiring hospitalization or leading to death after YF vaccination was 3.5 per 100,000 among people 65 to 75 years of age and 9.1 per 100,000 for people ≥75 years. For a rough comparison, the risk for vaccine-associated paralytic poliomyelitis due to oral polio vaccine was estimated as 1 per 2.5 million (22,23). A review of a passive surveillance system in the United Kingdom that receives reports from primary-care physicians also found a similar increase in SyAEs among elderly YF vaccine recipients (unpub. data).

Close examination of the two index cases and two additional deaths that followed YF vaccination shows four cases with similar clinical presentations, all of which share important characteristics with viscerotropic wild-type YF infection (18). Clinical presentations were characterized by fever, myalgia, headache, and confusion rapidly progressing to a multisystemic illness and death in three of the patients. The vaccine strain of YF virus was isolated from the serum of two patients and the cerebrospinal fluid of one. Sequence analysis of these isolates and the elevated antibody titers suggest an overwhelming infection caused by the selective amplification of a mutated virus subpopulation. The temporal relationship between severe illness and YF vaccination, the similar clinical presentations, and the laboratory results favor the hypothesis that these adverse events are causally related to YF vaccine. Recently, three cases of similar systemic adverse events after YF vaccination resulting in death were reported: two from Brazil, patients ages 5 and 22 years (Brazilian 17DD vaccine) and one from Australia, patient 56 years old (17D-204 vaccine) (17,19).

An increased risk for severe disease due to the vaccine strain of *Yellow fever virus* among older YF vaccine recipients is biologically plausible. Numerous reports and studies have shown that deaths and severe illnesses occur more frequently among the elderly with other flaviviral infections (e.g., West Nile encephalitis, Japanese encephalitis, Saint Louis encephalitis, Murray Valley encephalitis, and tick-borne encephalitis), while these infections are more likely to be self-limited in children (24-27). Similarly, investigations of YF outbreaks in the 1930s and 1940s found increased case-fatality rates among the oldest patients (28-30).

This study has several limitations. Rates calculated from VAERS data have to be interpreted with caution because of the problems inherent in a passive reporting system. Estimates of adverse events based on VAERS reports are likely to underestimate actual events (31). Our data,

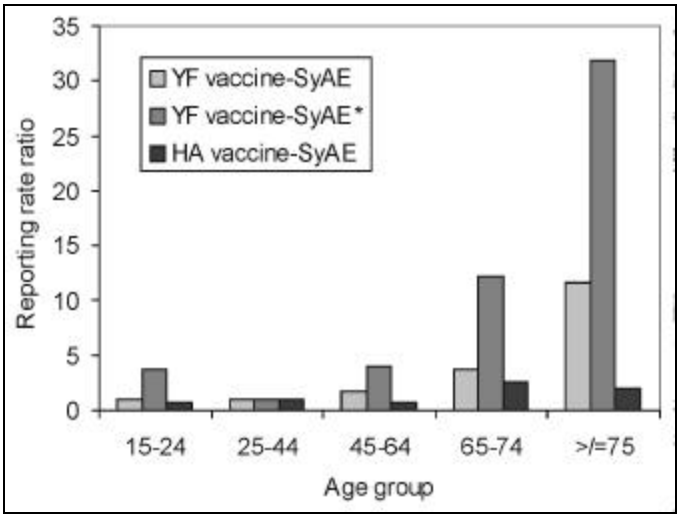


Figure 3. Reporting rate ratios for systemic adverse events (SyAE) and serious adverse events (SyAE*) after yellow fever (YF) vaccination and hepatitis A (HA) vaccination.

therefore, may reflect minimum estimates of these adverse events. Age-related reporting bias may also have influenced our results. Age-related reporting bias would decrease the significance of the reporting rate ratios only if SyAEs, particularly those leading to hospitalization and death, among vaccinees <65 years of age were reported less often than among vaccinees ≥65 years of age.

Another important limitation is that the estimated age distribution of travelers from the GeoSentinel clinics receiving YF vaccine in 1998 was assumed to apply for the entire study (1990 to 1998) and to be generalizable to the entire United States. However, if as many suspect, the proportion of elderly travelers has increased in recent years, this extrapolation will have overestimated the number of older travelers and have the effect of underestimating the reporting rate and reporting rate ratio of adverse events in the elderly. We excluded data on children <15 years of age, which caused a slight, proportionate increase in the denominator for the remaining vaccine recipients and a slight underestimate of the reporting rate for adverse events in all other age groups. Also, the calculation of denominators relied on assuming that the increase in vaccines administered between 1995 and 1996 held for 1990 to 1994.

An additional limitation is that age-specific SyAEs to YF vaccine may reflect an age-related response to vaccines in general or an increased amount of background illness in the elderly. Analysis of VAERS reports for SyAEs to HA vaccine did not support this conclusion; however, HA vaccine is an inactivated vaccine, and another live, attenuated vaccine would have served as a better control. We attempted to look at adverse events reported for oral typhoid vaccine, but the limited number of VAERS reports precluded a quantitative analysis.

Finally, although severe illness occurred days after YF vaccination in the cases we investigated, this temporal association does not prove causality. Definitive clinical or pathologic evidence identifying YF vaccine as the cause of severe illness or death for most cases reported to VAERS is lacking and is not routinely part of this surveillance system.

YF remains an important cause of severe illness and death in tropical South America and sub-Saharan Africa. In recent years, *Aedes aegypti*, the mosquito vector of urban YF, has reestablished itself in South America, increasing the likelihood of large, explosive outbreaks, and in both South America and sub-Saharan Africa, the number and size of outbreaks have increased in the last 20 years (32-34). Concomitant with these changes in the distribution of the vector and ongoing outbreaks, travel from the United States to disease-endemic regions has increased substantially (35). Quantitative risk assessments of YF among travelers to disease-endemic areas have not been done; however, the risk for acquiring YF has been highlighted by the recent deaths of four unvaccinated travelers due to YF imported to Europe and the United States (36-40).

The 17D YF vaccine has a long history of reported safety and efficacy and has played an important role in YF control, one of the public health triumphs of the 20th century. Age-specific recommendations and production standards for this important vaccine have been modified as a result of safety and efficacy issues that have become apparent with increased use (1,2,4,41,42). These modifications have preserved the vaccine as a vital tool for disease prevention and control. Defining the risk for adverse events among elderly vaccine recipients is an extension of these important efforts.

## Conclusion

This study provides data quantifying the relative reporting ratios of SyAE following YF vaccination among people ≥65 years of age in the United States. However, several issues must be addressed before any changes or restrictions to YF vaccine recommendations are proposed.

These SyAEs are still relatively rare (2.4 per 100,000 doses) in the United States, where an estimated 200,000 doses are given annually. The risk for unvaccinated travelers acquiring YF remains undefined; so risk-benefit estimates of YF vaccine are difficult to develop. In the absence of this important information, we suggest the following steps. Our observations should be confirmed by studies in different populations. Enhanced surveillance for systemic adverse events following YF vaccination should be introduced at U.S. certified vaccination centers and in other countries where YF vaccine is used. This enhanced surveillance should be combined with prospective follow-up that includes appropriate clinical, epidemiologic, and laboratory assessments of cases, biologic specimens, and vaccine or vaccine lots. In addition, epidemiologic studies should be designed to explore both host- and vaccine-specific factors associated with systemic adverse events (43).

In the interim, elderly YF vaccine recipients and their health-care providers should be cautioned about the possible risks of vaccination. Travel itineraries should be scrutinized, and the vaccine given only to those traveling to areas that report YF or are in the YF-endemic zone.

YF causes serious, life-threatening infections, and the vaccine is highly effective. The virus is responsible for substantial morbidity and death in disease-endemic areas and until more definitive evidence of vaccine-related adverse events is accumulated, the benefit-risk ratio of mass vaccination in YF-endemic countries favors continuation of a universal vaccine policy under the Expanded Programme on Immunization. Meanwhile, efforts to enhance our understanding of both the risks and benefits of YF vaccine and refine its use to maximize its safety and effectiveness should be accelerated.

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# Rapid Identification of *Bordetella pertussis* Pertactin Gene Variants Using LightCycler Real-Time Polymerase Chain Reaction Combined with Melting Curve Analysis and Gel Electrophoresis

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Recently, eight allelic variants of the pertactin gene (*prn1-8*) have been characterized in *Bordetella pertussis* strains isolated in Europe and the United States. It has been suggested that the divergence of the pertactin types of clinical isolates from those of the *B. pertussis* vaccine strains is a result of vaccine-driven evolution. Sequencing of the *prn,* which is relatively time-consuming, has so far been the only method for the differentiation of *prn* types. We have developed a rapid real-time polymerase chain reaction assay suitable for large-scale screening of the *prn* type of the circulating strains. This method correctly identified the *prn* type of all tested 41 clinical isolates and two Finnish vaccine strains. The method is simple and reliable and provides an alternative for sequencing in pertussis research.

*Bordetella pertussis* is the causative agent of pertussis (whooping cough), which is increasing in incidence in several countries despite high vaccination rates (1-5). One explanation for the increase might be the adaptation of *B. pertussis* bacteria to vaccine-induced immunity. Pertactin, a 69-kDa outer membrane protein, is an important virulence factor of *B. pertussis*. Because pertactin elicits protective immunity in animals and humans during vaccination (6-9), this protein is included in most new acellular pertussis vaccines. Pertactin contains two immunodominant regions, regions 1 and 2, comprising repeating units of five (GGxxP) or three (PQP) amino acids, respectively (10-12). It has been suggested that the number of the units is regulated through genetic recombination (12). Recently, eight allelic variants of the pertactin gene (*prn1-8*) have been characterized in *B. pertussis* strains isolated in Europe and the United States (12-16). Most of the allelic variation in *prn*1-5 are restricted to region 1, whereas *prn6-8* also show variation in region 2 (13). *prn1-3* are the predominant types, representing >90% of tested clinical isolates (12-16), whereas vaccine strains have exclusively *prn1* (12,14-16). Of 92 strains isolated between 1989 and 1999 in the United States, 30% harbored *prn1* and 70% *prn2* (14). In the Netherlands and Finland, approximately 10% of clinical strains isolated in the 1990s harbored *prn1* and 90%, *prn2* or *prn3* (12,15).

So far, the only means of determining the pertactin type has been polymerase chain reaction (PCR)-based sequencing of the *prn* gene, a relatively time-consuming and expensive method. To monitor the variation of clinical isolates on a large scale, a rapid and simple method is needed. The recent applications of fluorescence techniques to PCR allow real-time monitoring of accumulation of the amplified product and accurate analysis of the melting temperatures of either the amplified product itself or the attached hybridization probes (17-21). In the hybridization probe format the two independent, nonextendible, single-labeled oligonucleotide probes hybridize adjacently on the amplicon internal to the flanking PCR primers. After excitation by the light-emitting diode, a fluorescence resonance energy transfer (FRET) occurs from the donor dye to the acceptor dye, increasing the signal emitted by the acceptor dye (22).

We developed a simple method to characterize the pertactin variants (Figure 1). The strains with the frequent types, *prn1-5,* were first differentiated from strains with the rare types, *prn6-8,* by a real-time allele-specific amplification (ASA) assay. Strains representing *prn1-5* were further identified by a real-time PCR combined with the melting curve analysis of FRET probes and gel electrophoresis. Results were compared to those obtained by sequencing (15). The speed and simplicity of this approach make it an advantageous alternative to conventional sequencing of the *prn* gene.

## Materials and Methods

### Bacterial Strains and DNA Sequencing

Forty-one clinical *B. pertussis* isolates and 2 Finnish vaccine strains were selected from the strain collection of the Pertussis Reference Laboratory, National Public Health Institute, Turku, Finland. All 41 clinical isolates originated from Finland and were isolated from 1956 to 1996. The *prn*

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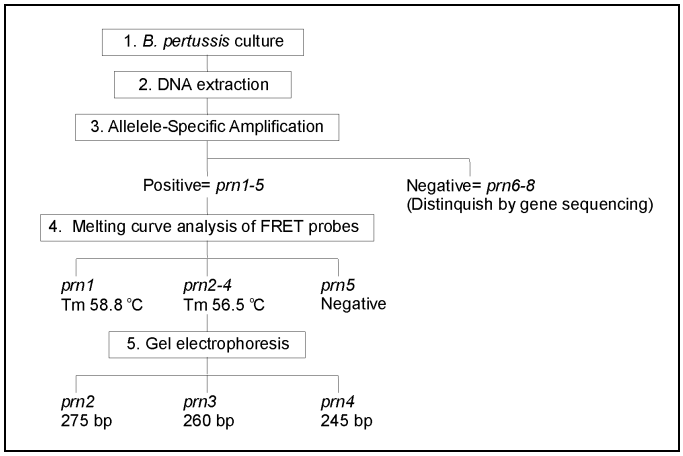


Figure 1. Workflow for typing *prn* alleles. The allele-specific amplification (ASA) assay (step number 3) and the fluorescence resonance energy transfer (FRET) probe assay (step number 4) each require approximately 1 hour.

genes of these isolates and strains have been previously sequenced, and the *prn* sequences of 38 were published earlier (15). All the Finnish strains represented *prn1-4*. Strains B935 (AJ011016), 18323 (AJ132095), B567 (AJ133784), and B1092 (AJ133245) harbor *prn*5, *prn6*, *prn7*, and *prn8*, respectively.

Bacteria were cultivated on Regan-Lowe medium containing charcoal agar and defibrinated horse blood at 35°C for 3 days (23). Bacterial colonies on the plates were harvested for isolation of DNA. PCR-based sequencing was done as described previously (12).

### Primers and Probes

Primers for real-time ASA and the FRET probe assay were designed on the basis of the published sequence of the *prn* genes (10,12,15,16) and synthesized at Eurogentech, Seraing, Belgium (Table 1). Of primers used in ASA assay (Table 1) (Figures 2,3), QJF3 contained a specific mismatch G at the 3' end that did not complement the published sequences of any *prn* type. The T (boldfaced) at the 3'end of QJF3 (corresponding to the nucleotide 1595) was complementary to *prn1-5* but not to *prn6-8* to permit preferential amplification of the former types. The two mismatches at the 3'end of QJF3 would guarantee the absence of PCR amplification when the sequences of *prn6-8* are used as targets (24,25). The primers QJF3 and QJR1 define a 72-bp long PCR product. The primers QH8F' and QH2R used in the FRET probe assay define a 260-bp long PCR product. Based on earlier sequencing data, the calculated lengths of the PCR products were 260 bp, 275 bp, 260 bp, 245 bp, and 245 bp for *prn1*, *2*, *3*, *4*, and *5*, respectively. The FRET hybridization probes QJ1 and QJ2 were designed on the basis of the sequence of *prn1* to differentiate *prn1* from *prn3* (Table 1) (Figures 3,4). The boldfaced T of probe QJ2 is complementary to C to T transition specific for *prn*1 (corresponding to nucleotide 828) (13). Binding of probe QJ2 to *prn5* (compared to the *prn1-4*) will be hampered since no complementary sequence to the probe is available on *prn5* (Figure 4A, B). Probes were synthesized at TIB Molbiol, Berlin, Germany. QJ1 (used as the donor probe in FRET technology) was labeled with fluorescein at the 3' end. QJ2 was labeled with LightCycler Red 640 at the 5'end and phosphorylated at the 3' end; this was used as the acceptor probe in the FRET (Table 1) (Figure 4A).

Table 1. Primers and probes used in study of *Bordetella pertussis* pertactin gene variants

| Primer/ probe | Sequence (5'-3')[a] | Position[b] |
|---|---|---|
| QJF3[c] | GCT GGT GCA GAC GCC AG**T** | 1578-1595 |
| QJR1[c] | CCG ATA TCG ACC TTG CC | 1649-1633 |
| QH8F[d] | CTG CAG CGC GCG ACG ATA | 757-774 |
| QH2R[d] | ATT GCC GTG CGG TGC GGA CAA | 1026-1006 |
| QJ1[e] | CCG GCG GTG CGG TTC C-**F** | 809-824 |
| QJ2[e] | **LC Red 640**-CGG **T**GG TGC GGT TCC C-**P** | 825-840 |

[a]Modifications of primer or probe are boldfaced or underlined.
[b]Position numbers indicate the position of bases relative to the first start codon of *prn1*.
[c]Primers used in the real-time allele-specific amplification. QJF3 contained a specific mismatch G (underlined) at the 3' end that does not complement the published sequences of any *prn* type. The T (boldfaced) at the 3' end (corresponding to the nucleotide 1595) is complementary to *prn1-5*. Primer QJF3 has two mismatches with *prn6-8*.
[d]Primers used in fluorescence resonance energy transfer (FRET) probe assay.
[e]Probes used in FRET probe assay. Boldfaced T is complementary to C to T transition specific for *prn1.* QJ1 was labeled with fluorescein at the 3' end and QJ2 with LC Red at 5' end and phosphorylated at the 3' end.

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                                        1595
QJF3        5´GCT GGT GCA GAC GCC AGT 3´

Prn 1-5       GCT GGT GCA GAC GCC ACT
Prn 6-8       --- --- --- --- --- --G
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Figure 2. Partial sequence of the *prn* gene of *Bordetella pertussis*, showing the position of QJF3 primer. Consensus bases are shown with dashes, and the mismatched bases in the primer are underlined.

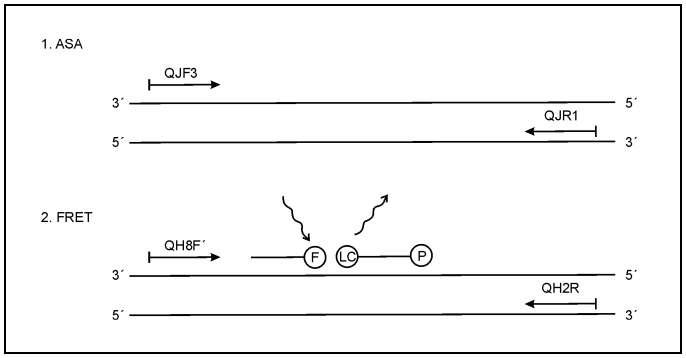


Figure 3. Schematic structure of the *prn1* gene, showing the position of the primers used in the allele-specific amplification assay (1.), and the primers and probes used in the fluorescence resonance energy transfer probe assay (2). Proportions of the gene are not drawn to scale. F = fluorescein; LC = LC red 640; and P = phosphate.

### DNA Preparation

DNA was extracted from bacterial colonies by using the DNA Isolation Kit for Blood/Bone Marrow/Tissue (Roche Diagnostics, Mannheim, Germany) according to the

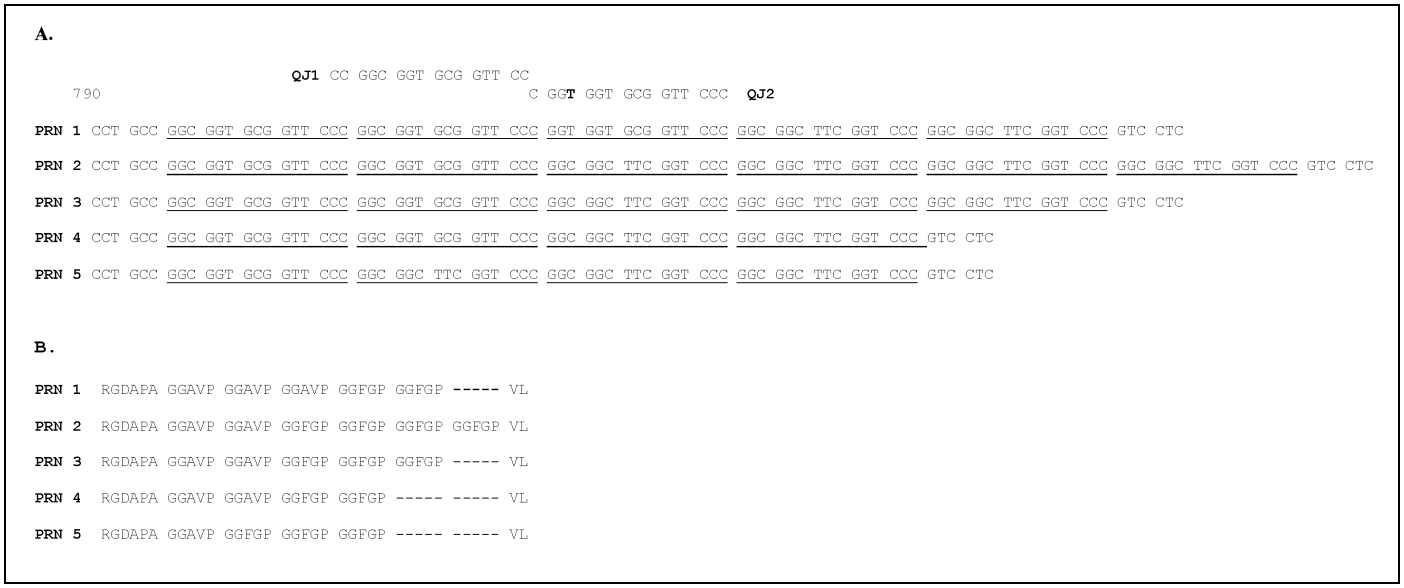


Figure 4A. Nucleotide sequences of polymorphic regions of different types of the *prn* gene and the sequences of the fluorescence resonance energy transfer probes aligned to their hybridization positions in the *prn* gene. Number 790 refers to the position of bases relative to the first start codon of *prn1*. Underlined regions represent repeats in the sequence. B. Amino acid sequences of polymorphic regions of different pertactin types. Dashes indicate caps in the sequence.

manufacturer's instructions. Extracted DNA concentrations were measured with a GeneQuant spectrophotometer (Pharmacia Biotech, NJ, USA). DNA concentrations in all samples were adjusted to 3 ng/µL. DNA preparations were stored at -20°C.

### Allele-Specific Amplification (ASA)

ASA PCR, which distinguishes between *prn1-5* and *prn6-8,* was performed in a fluorescence temperature cycler (LightCycler, Roche). The PCR reaction mixture was optimized for the LightCycler and amplified according to the manufacturer's protocol. The final volume of 20 µL contained 2 µL of LightCycler-DNA Master SYBR Green I (containing Taq DNA polymerase, reaction buffer, deoxynucleoside triphosphate (dNTP) mix and dsDNA binding dye SYBR Green I), 4 mM $MgCl_2$ (Roche), 8 pmol of the primers QJF3 and QJR1, 5% dimethyl sulfoxide (Merck, Darmstadt, Germany), and 2 µL of 3-ng/µL sample DNA. A negative control without DNA and a positive control that contained 6 ng of the DNA from strain 1772 (*prn1*) were included in each run. The amplification protocol consisted of the initial denaturation step at 94°C for 30 seconds, 30 cycles of denaturation at 95°C for 1 second, annealing at 62°C for 5 seconds, and extension at 72°C for 4 seconds. The temperature transition rate was 20°C per second. Fluorescence was measured at the end of each extension step at 530 nm. The increase in the fluorescence signal correlates to the accumulation of PCR product (19,22).

After amplification, melting curve analysis of the PCR product was used to differentiate between specific and nonspecific amplification products. Melting curve was acquired by heating the product at 20°C/seconds to 95°C, cooling it at 20°C/seconds to 55°C for 30 seconds, and slowly heating it at 0.1°C/seconds to 94°C under continuous fluorescence monitoring. Melting curve analysis was accomplished with LightCycler software. As the temperature reaches the specific Tm of the PCR product, the double-stranded product is rendered into the single-stranded form. A rapid loss of fluorescence can be observed as the double-stranded DNA binding dye SYBR green I detaches from the PCR products. The change in fluorescence signal intensity is then plotted as the negative derivative of fluorescence versus temperature (–dF/dT vs T graphs) to obtain the characteristic melting peaks. In constant reaction conditions (salt concentration and the like), the position of the melting curve peak (Tm) is a function of the GC/AT ratio, length, and nucleotide sequence of the PCR product (26). Melting curve analysis has been successfully used in the differentiation of PCR products with a difference of even <2°C in the Tm (18,26).

### Hybridization Probe Assay

The hybridization probe assay was carried out by using the FRET probe format of LightCycler. The PCR reaction mixture was optimized for the LightCycler and amplified according to the manufacturer's protocol. The final volume of 20 µL contained 2 µL of LightCycler-DNA Master Hybridization Probes (containing Taq DNA polymerase, reaction buffer, and dNTP mix), 3 mM $MgCl_2$ (Roche), 1.5 pmol of the FRET probes QJ1 and QJ2, 8 pmol of the primers QH8F' and QH2R, 220 ng of TaqStart antibody (ClonTech, CA, USA), 10% dimethyl sulfoxide (Merck), and 2 µL of sample DNA. A negative control without DNA and two positive controls representing *prn1* and *3* were included in each run. The temperature profile of the real-time PCR included an initial denaturation step at 94°C for 120 seconds followed by 40 cycles of denaturation at 94°C for 2 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 12 seconds. The temperature transition rate was 20°C/s. Fluorescence was measured at 640 nm at the end of the annealing step of each cycle to monitor the accumulation of PCR product.

After amplification, a melting curve was acquired by heating the product at 20°C/seconds to 95°C, cooling it at 20°C/seconds to 42°C for 120 seconds, and slowly heating it at 0.1°C/seconds to 80°C under continuous fluorescence monitoring. Melting curve analysis was accomplished using LightCycler software. In the hybridization probe format, the rapid loss of fluorescence is observed when the temperature

reaches the Tm of the probes. The two adjacently bound probes dissociate from their complementary target, which prevents the fluorescence resonance energy transfer. Melting curve analysis allowed us to discriminate the specific binding of the hybridization probes to the amplified segment of the *prn1* from their less specific binding to the amplified segments of the other *prn* types.

### Analysis of the FRET Hybridization Probe Assay PCR Products by Gel Electrophoresis

A 20-µL volume of LightCycler PCR product from the hybridization probe assay was removed from the capillary by removing the cap, placing the capillary upside down in an empty Eppendorf tube, and centrifuging for 5 seconds. A 10-µL volume of the PCR product was run (100 V for 3 hours) in a 3% molecular screening (MS) agarose gel (Roche Diagnostics) together with a 100-bp DNA ladder (Amersham). According to the manufacturer, the resolution characteristics of MS agarose enable separation of fragments that differ in size by as little as 4 bp. After being stained with ethidium bromide, the bands in the gel were visualized and photographed under UV light. To avoid PCR contamination, three separate rooms were used for preparing the PCR mixtures, performing PCR reactions, and analyzing PCR products.

### Statistical Analysis

The Student *t* test was used to analyze statistical significance. All p values corresponded to two-tailed tests, and p <0.05 was considered significant.

## Results

### Differentiation of *prn1-5* from *6-8*

The ASA assay was used as a screening method to differentiate the frequent *prn* types *(prn1-5*) from rare types (*prn6-8*). When compared to previous sequencing data (12), all type strains and clinical isolates were correctly categorized by this assay. The mean Tm of the PCR products derived from the *prn1-5* strains was 83.4°C (standard deviation [SD] 0,54) (Figure 5). There was no specific amplification from the strains with *prn6-8*. The nonspecific products, such as primer dimers, melt below 80°C and were differentiated from the specific products by the melting curve analysis. These results were also confirmed by gel electrophoresis.

### Melting Curve Analysis of Hybridization Probe Assay

Strains that were found to harbor *prn1-5* in the screening were further analyzed by the combination of hybridization probe assay and gel electrophoresis. FRET probes were designed as complementary to the *prn1* gene. The Tms and results of the melting curve analyses of the strains with the *prn1* gene differed markedly from those of strains with *prn2*, *prn3*, and *prn4* (Figure 6). All nine strains harboring the *prn1* gene showed an abrupt decrease in the fluorescence signal (Table 2) and a melting peak (Figure 6) at 58.78°C (SD 0.26). The corresponding melting peak was observed at the same temperature in all nine *prn1* strains. Although probes did not remain bound to PCR products of *prn2*, *prn3*, and *prn4* when the fluorescence signal was measured at the end of the annealing cycle at 55°C, probes bound to those products at the beginning of the melting analysis at 42°C, and Tm and the area under the melting curve (AUC) could also

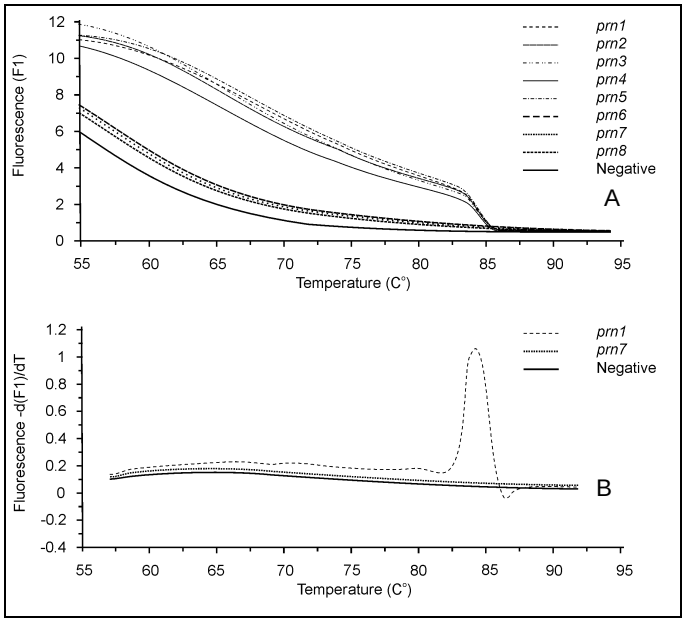


Figure 5A. Melting curves from the allele-specific amplification assay, showing the presence of amplified products from *prn1-5* and the absence of amplification from *prn6-8* and the negative control. B. Corresponding melting peaks derived from the melting curve. *Prn1* represents the *prn1-5* types; *prn7* represents *prn6-8* types and the negative control.

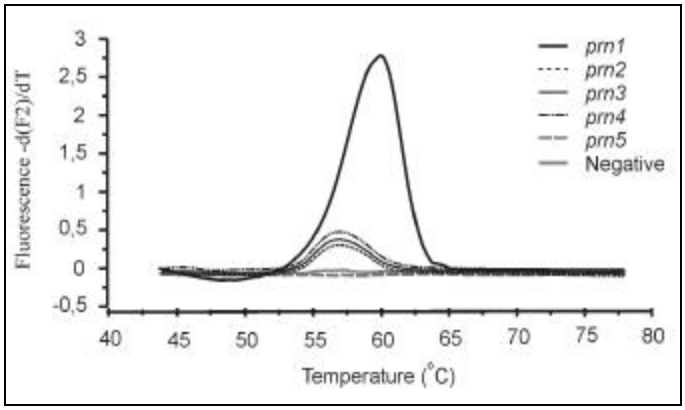


Figure 6. Curves showing the dissociation of fluorescence resonance energy transfer probe assay probes from the polymerase chain reaction products of different *prn* types. Negative control includes all reagents but no template DNA.

be determined for these products. As expected, the Tm of FRET probes bound to the PCR products derived from *prn2*, *prn3*, and *prn4* was 2°C lower (56.49°C) than that of the FRET probes bound to the PCR product of *prn1* (all p values for differences between the Tm of *prn1* and that of the other *prn* types were <0.0001) (Table 2) (Figure 6). The AUC of the hybridization probe melting curve of *prn1* was approximately 20 times larger than that of the melting curve of the *prn2*, *prn3*, and *prn4* (all p values <0.0001) (Table 2) (Figure 6). There was no detectable binding of FRET probes to the PCR products derived from the *prn5* strain (Figure 6). DNA isolated from the strain with *prn5* (together with DNAs from strains representing *prn1-4* that served as controls) was tested in triplicate and with different DNA concentrations with the same result. In contrast to *prn2-4*, there was no measurable melting temperature and no AUC from the DNA isolated from the strain with *prn5*, although the PCR product was seen (245-bp long on the electrophoresis gel) (Figure

Table 2. Comparison of the results from sequencing and the hybridization probe assay in the determination of the pertactin gene type of *Bordetella pertussis* strains

| Pertactin allele type[a] | No. of isolates | Melting temperature | | Melting curve | |
|---|---|---|---|---|---|
| | | Mean | SD | Area | SD |
| 1 | 9 | 58.78[b] | 0.26 | 21.64[b] | 5.64 |
| 2 | 25 | 56.31 | 0.44 | 1.25 | 0.30 |
| 3 | 4 | 56.59 | 0.09 | 1.35 | 0.26 |
| 4 | 5 | 56.57 | 0.29 | 1.43 | 0.25 |
| 5 | 1 | - | - | - | - |

[a]Allele type determined by sequencing. Of the strains representing *prn1-4*, 41 were Finnish clinical isolates, and 2 were Finnish vaccine strains.
[b]All P values were <0.0001 when *prn1* was compared to *prn2*, *prn3*, or *prn4*.

7). In this setting, therefore, a sample that remained totally negative (no measurable melting temperature and no AUC) in the hybridization probe assay but was characterized as *prn1-5* type strain by the ASA assay was considered to harbor *prn5*.

### Gel Electrophoresis of PCR Products

Calculated sizes of PCR products were 260 bp, 275 bp, 260 bp, 245 bp, and 245 bp for *prn1* to *5*, respectively. PCR products of the different *prn* types behaved in gel electrophoresis as expected on the basis of their calculated sizes (Figure 7). Thus, *prn2*, *prn3*, and *prn4* could be easily differentiated by gel electrophoresis when the *prn1* and *prn5* were identified by the melting curve analysis of hybridization probes.

### Identification of the *prn* Type of *B. pertussis* Isolates and Vaccine Strains

The *prn* types of all tested 41 Finnish clinical *B. pertussis* isolates and 2 Finnish vaccine strains were identified correctly when compared to types defined by sequencing (Table 2). None of these strains was found to harbor *prn5-8*.

## Discussion

Real-time PCR combined with melting curve analysis of FRET probes and gel electrophoresis of PCR products proved to be an alternative to sequencing in the determination of the pertactin gene types of *B. pertussis*. The method was reliable and accurate, as evidenced by the correct identification of the *prn* type of all tested 41 clinical *B. pertussis* isolates, two vaccine strains, and the four reference strains. The advantage of this approach over sequencing is that the whole procedure from nucleic acid extraction to gel electrophoresis can be completed within 1 day. The disadvantages of the method are that the novel genotypes can be missed and the method does not differentiate *prn6-8* from each other.

The low intra- and inter-assay variation coefficients of melting temperatures show that the technical principles of LightCycler allow consistent temperature and fluorescence measurement conditions for the reaction capillaries. This is a definite advantage over the corresponding instruments using the microwell plate format, which requires intrinsic correction to compensate for technical variation between reaction wells.

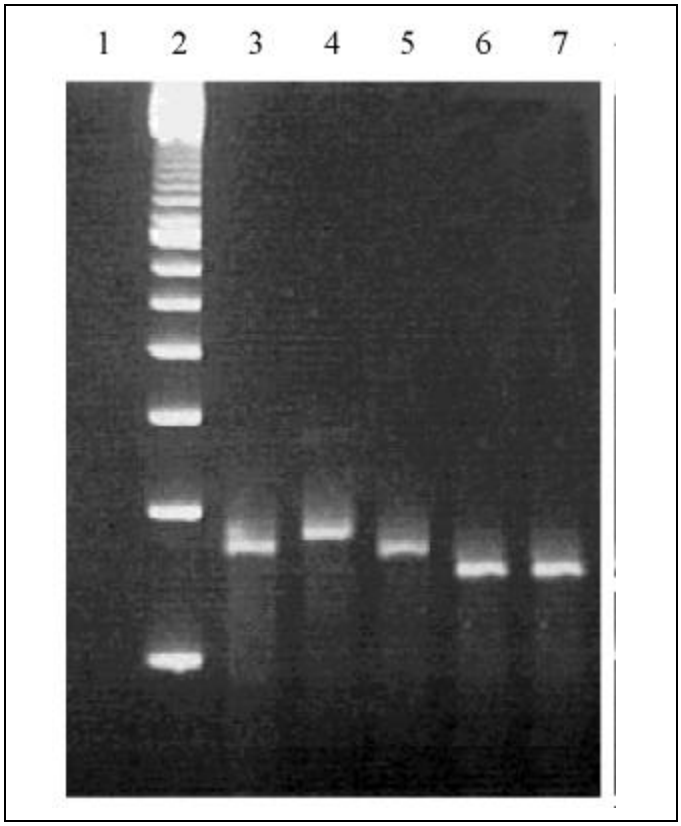


Figure 7. Ethidium bromide stained 3% molecular screening agarose gel containing *Bordetella pertussis* DNA amplified with primers QH8F' and QH2R. Lanes: 1, negative control including all reagents but no template DNA; 2, 100-bp ladder; 3, *B. pertussis* strain 1772 of type *prn1* (260 bp); 4, *Bordetella pertussis* clinical isolate of type *prn2* (275 bp); 5, *B. pertussis* clinical isolate of type *prn3* (260 bp); 6, *B. pertussis* clinical isolate of type *prn4* (245 bp); and 7, *B. pertussis* type *prn5* (245 bp).

In this study, a real-time ASA assay was used as a screening method to first differentiate the frequent *prn* types *prn1-5* from the rare types *prn6-8* (13). In ASA assay, when primers designed to be specific for either wild-type or the mutant allele are used, results depend on the presence or absence of amplification. In this study the PCR amplification by the primers specifically designed for the allele *prn1-5* took place with DNAs extracted from *prn1-5* strains but not with those isolated from strains representing *prn6-8*. When ASA reactions occur in a fluorescence thermal cycler such as LightCycler, accumulation of the PCR product can be monitored in real-time. The analysis of specific melting temperatures further confirms the identity of the amplified products.

The FRET probes were specifically designed to identify *prn1* so that the strains representing the vaccine type *prn* could be rapidly detected. The FRET probes also enabled differentiation of *prn1* from *prn3*, the two *prn* types that cannot be differentiated on the basis of the size of the PCR product. The *prn1* sequence contains an additional C to T transition (corresponding to nucleotide 828) that makes it possible to design probes that are specific for just one *prn* type. The probes did not bind to the PCR products of *prn2*, *prn3*, *prn4*, and *prn5* in the fluorescence measurement phase of the PCR cycle at 55°C. Therefore, no signal was obtained in the real-time PCR from DNA of bacteria having these *prn* types. However, probes did bind to the PCR products of *prn2*, *3*,

and *4* at the beginning of the melting analysis at 42°C, and the Tm and AUC values could also be determined for these products. Their Tm was >2°C lower and AUC approximately 20 times smaller than those of the PCR product of *prn1*. These differences clearly reflect the lower sequence compatibility of the probes for *prn2*, *prn3*, and *prn4* than for *prn1*. As expected, the Tms of *prn2*, *prn3*, and *prn4* were almost identical because the target sequences of the probes in these *prn* types were the same. *Prn5* has the same number of repeats as *prn4* does, which makes their PCR products the same length. However, the difference between these two is that *prn4* has two repeats of "GGAVP," as do *prn2* and *prn3*, whereas *prn5* has only one such repeat. *Prn5* is the only type to which there was no detectable binding of the FRET probes during the amplification phase or the melting curve analysis. This property could be used to differentiate *prn5* from *prn4*.

The difference in size of the PCR products derived from the *prn2*, *prn3*, and *prn4* (or *prn*5) is 15 bp. To assure the correct identification of the pertactin types, a gel with a high-resolution power is needed for the electrophoresis. In this study molecular screening agarose gel was used, since the resolution characteristics of this agarose enable separation of fragments that differ in size by as little as 4 bp.

Recent data suggest that *B. pertussis* strains having different *prn* types are circulating in Europe and the United States. The predominant types representing >90% of the tested clinical isolates are *prn1-3* (12-16).

In the United States, all strains isolated before 1974 harbored *prn1* (14), the *prn* type of the strains included in conventional whole-cell vaccines and in the new acellular vaccines. However, nonvaccine *prn* types gradually replaced the vaccine types in later years, and approximately 30% of strains isolated between 1989 and 1999 were *prn1*. Similar trends of frequency in strain types were also seen in European countries. The method described here is suitable for monitoring the frequency of the strain types of clinical isolates. The strains representing *prn1* can be detected by running the two PCR reactions (ASA and hybridization probe assay), and the results can be obtained within 2 hours. When the strains that do not represent vaccine strain type need to be clarified, gel electrophoresis of PCR products can be performed. To combat pertussis and to design more effective vaccines, the variation of pertactin and other virulence factors of the *B. pertussis* strains circulating in the population has to be monitored. It is possible that the antigenic variation is a result of vaccine-driven evolution, possibly protecting the bacteria from the attacks of the host's specific immune response. The method described here is convenient for large-scale screening of pertactin variation in *B. pertussis* isolates. Data obtained by large-scale screening provide the epidemiologic picture of the circulating strains. This information may further help in vaccine formulation, which enables more efficient protection against pertussis. It is also possible to use a similar approach to detect the variation in pertussis toxin gene that has been already characterized, or in studies on genetic variation in any species.

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# Modeling Potential Responses to Smallpox as a Bioterrorist Weapon

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We constructed a mathematical model to describe the spread of smallpox after a deliberate release of the virus. Assuming 100 persons initially infected and 3 persons infected per infectious person, quarantine alone could stop disease transmission but would require a minimum daily removal rate of 50% of those with overt symptoms. Vaccination would stop the outbreak within 365 days after release only if disease transmission were reduced to $\geq$0.85 persons infected per infectious person. A combined vaccination and quarantine campaign could stop an outbreak if a daily quarantine rate of 25% were achieved and vaccination reduced smallpox transmission by $\geq$33%. In such a scenario, approximately 4,200 cases would occur and 365 days would be needed to stop the outbreak. Historical data indicate that a median of 2,155 smallpox vaccine doses per case were given to stop outbreaks, implying that a stockpile of 40 million doses should be adequate.

Recent papers have speculated about the use of smallpox as a biological weapon (1-5). If we assume such a risk, there is concern about the need for preparations to limit and prevent the spread of smallpox after a deliberate release of the virus. Studies of smallpox control and eradication efforts (6-8) identified two available types of interventions: vaccination of those at risk from infection, quarantine, or both. Some studies have provided estimates of the potential numbers that could be infected (1,3,5) and the number of vaccine doses that should be stockpiled (5); however, they did not provide details of how these estimates were calculated. Further, none of these articles examined how quarantine of infected persons may help halt transmission of smallpox.

Crucial questions that remained unanswered include—How can we calculate the number of doses of smallpox vaccine to be stockpiled? Can quarantine contribute to control efforts? How effective does quarantine have to be to reduce transmission? We present a mathematical model that helps answer these and other questions.

## Methods

We constructed a mathematical model to meet the following objectives: 1) describe the spread of smallpox through a susceptible population, calculating daily (new-onset) and cumulative cases; 2) readily accommodate changes in input values, such as the number of persons infected per infectious person (i.e., rate of transmission) and the number of persons initially infected; 3) examine the impact of quarantine and vaccination, alone and in combination, on the spread of smallpox; and 4) estimate the number of doses of smallpox vaccine that should be stockpiled as part of readiness plans.

Despite numerous reports of mathematical models of infectious diseases (9-14), few such models describe the spread of smallpox. Frauenthal (15) addressed the question of optimal level of smallpox vaccination. We constructed a Markov chain model (16) to describe the spread of smallpox through a susceptible population (objective 1), using a computer-based spreadsheet program (Excel97, Microsoft, Redmond, WA). The model describes four disease stages: incubating, prodromal, overtly symptomatic, and no longer infectious (Figure 1). The term "prodromal" indicates the preeruptive stage.[1] "Overtly symptomatic" refers to the period of disease when a rash or similar symptoms can be readily noted by even an untrained observer.[2] For each day after the release, the model calculates both the number of new cases and the cumulative total.

In the model, an infected person can only progress, from incubating to prodromal to overtly symptomatic, and cannot revert. The duration in days of a given disease stage is controlled by a probability function (Figure 2).

### Probable Durations of Each Disease Stage

When smallpox was endemic in human populations, the incubation period was often difficult to measure because many patients were exposed over several days (7,8). Fenner et al. (7) reviewed and summarized three reports in which the incubation period was calculated for 255 cases of variola major smallpox (the "classic" form). Just over 70% of these cases incubated 9 to 13 days, with an average of 11.5 days (range 7 to 19 days; median approximately 11 days; 5th

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[1]Others have suggested that the terms "preeruptive" or "initial" are more descriptively accurate of this stage (6). However, because "prodromal" is used in many standard textbooks (7,8,17), we will use this term.
[2]Prodromal rashes have been recorded, but they were considered to be uncommon occurrences, ". . . not more than 1 in 10." (17).

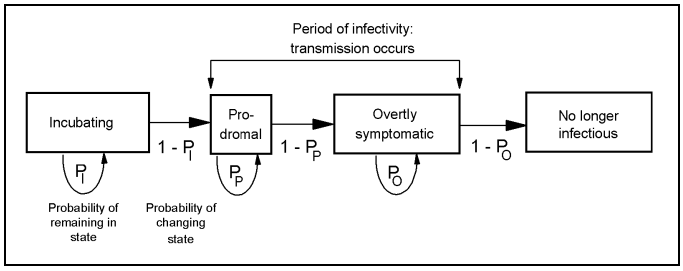


Figure 1. Schematic of the Markov-chain model used to model the movement of a person infected with smallpox through the four stages of disease. $P_I$ = probability of remaining in the incubating stage; $P_P$ = probability of remaining in the prodromal stage; and $P_O$ = probability of remaining in the overtly symptomatic stage. For each stage, the probabilities of remaining in that stage ($P_I$,$P_P$,$P_O$) are determined by a daily probability (Figure 2). Patients who have reached the fourth and final stage (no longer infectious) effectively drop out of the model. The "overtly symptomatic" stage refers to the period of disease when a person has a rash or similar symptoms that even an untrained observer can readily note. During the period of infectivity, the average number of persons infected per infectious patient is preset by the researchers. The days when transmissions occur are determined by a probability function.

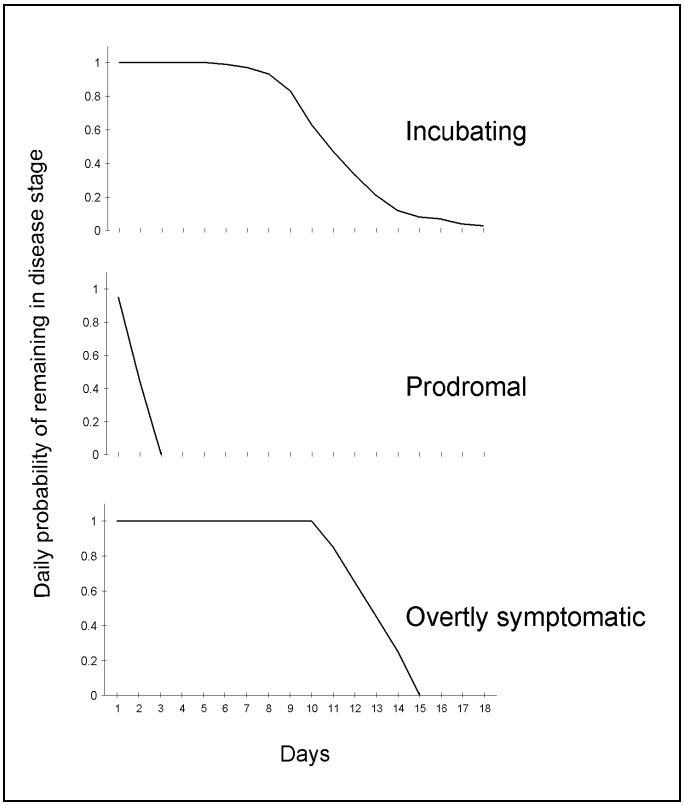


Figure 2. Probability functions associated with remaining in three smallpox disease stages. These reverse cumulative probability functions describe the probability on any defined day of a patient remaining in a disease stage during the next day. On any given day, the probability of moving from one stage to the next is 1 minus the probability of remaining in the stage.

percentile 8 days; and 95th percentile 14 days). Others have observed similar lengths of incubation. For example, by examining the time between onset and "brief and only possible contact with a known case," Singh (18) determined the possible length of incubation of six cases of smallpox (mean 11 days; median 12 days). Rao (6) used data from 50 first-generation cases to determine that the mean "fever-to-fever" (i.e., onset of fever to onset of fever) interval was 16 days (range 12 to 21 days for 80% of cases).

Using data from 115 cases in Europe (19), we constructed a reverse cumulative probability function to describe the probability of a person on a given day remaining in the state of incubation for the next day (Figure 2). The calculated mean was 11.7 incubating days (median approximately 11 days; 5th percentile 8 days; and 95th percentile 17 days). The function used can be altered to reflect other data sets or hypothesized functions. Further, the model can accept different transition probability functions for each day in the model.

The duration of the prodromal stage is variable and depends in part on the ability of the physician or patient to detect the first lesion (6). The onset of rash (the overtly symptomatic stage) typically occurs 48 to 72 hours after onset of fever, although some types of smallpox may have a prolonged prodromal stage of 4 to 6 days (6). Fenner et al. reviewed several data sources and used temperature data to report that the prodromal stage lasts an average of 3 days (7). Beyond these descriptions of the average or typical course of disease, no data are readily available documenting the probabilities associated with a longer prodromal stage (e.g., frequency data linking number of patients to number of days in the prodromal stage). Thus, we assumed a linear decline in the daily probability of remaining in the prodromal stage (Figure 2). The probabilities decline from 0.95 at the end of day 1 (i.e., a 95% chance that the patient will be in prodromal stage for another day) to 0.00 at the end of day 3 (i.e., absolute certainty that the prodromal stage will not last beyond day 3).

The average total time of illness (i.e., having some symptoms) is given in Fenner et al. (7) as 21 days, with scabbing on day 19. Allowing up to 3 days for the prodromal period (Figure 2) leaves an average of 16 days in the overtly symptomatic period in which a patient can infect others. Although scabs may contain infectious amounts of smallpox virus after the patient has fully recovered, we assumed that after scabbing, neither the patient nor the scabs will pose a substantial source of infection. The exact duration of illness is somewhat moot, as the likelihood of transmission declines after the first few days of overt symptoms. Thus, after some period, a person who is overtly symptomatic has a low probability of infecting a susceptible person. We assumed a probability of 1.00 (i.e., absolute certainty) of remaining the next day in the overtly symptomatic stage for the first 10 days in the stage. Including the prodromal stage, this corresponds to 12-15 days of illness (Figure 2). After 10 days, a patient's daily probability of remaining in the stage decreases linearly, so that 15 days after onset of symptoms the probability of remaining the next day in this stage is 0.00 (Figure 2). That is, after a maximum of 16 days in the overtly symptomatic stage, all patients will have progressed to the "no longer infectious" stage. Patients who have reached the fourth and final stage (no longer infectious) effectively drop out of the model. These probability functions can readily be changed (objective 2).

### Likelihood of Smallpox Transmission

Also described by a probability function is the likelihood of smallpox transmission during the infectious period. For a variety of reasons, the probability of transmission is likely to change during the period when an infected person is infectious. For example, persons with a high fever during the first

2 days of the prodromal stage (Figure 2) may voluntarily confine themselves to quarters, possibly limiting their opportunities to infect others. Limited data are available regarding changes in the probability of when an infection is transmitted, but Mack (19) and Rao (6) provide a time series of data involving 23 and 60 patients, respectively. Both data sets suggest that transmission is less likely during the prodromal stage (the first 3 days when a person is symptomatic) and that the probability of transmission is greatest between days 3 and 6 after a patient becomes infectious (Figure 3). This period is equivalent to the first to third days of onset of rash (overt symptoms). Both data sets (6,19) indicate that 70% to 80% of transmission is likely to occur in the first 9 days of the symptomatic period, and 90% of all transmission will have occurred in 10 to 13 days (Figure 3). In other words, by day 6 of overt symptoms (rash), approximately 75% of transmissions will have occurred, with 90% occurring within 7 to 10 days. For the model, we used the data from Mack (19) to describe the probabilities of when transmission will occur, from infectious to newly infected (Figure 3). Other data sets and probability functions can readily be substituted.

### Existing Immunity and Community Size

For simplicity, we assumed an unlimited supply of susceptible persons,[3] so that disease transmission will not be halted because of lack of susceptible persons. Although this scenario is unrealistic for modeling the natural spread of an infectious disease, it may be realistic for considering the initial spread of an infectious disease after deliberate infection of a small number of persons in a population with a relatively large proportion who are susceptible.

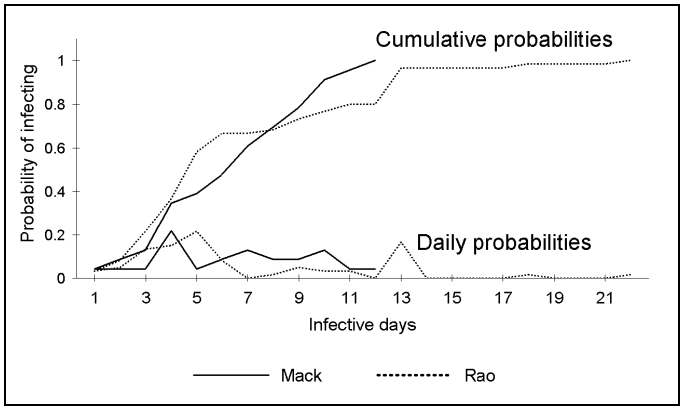


Figure 3. Daily and cumulative probabilities determining when an infectious person infects another person with smallpox (6,19). Day 1 of the infectious period is the first day of the prodromal stage. That is, we have interpreted the source data to reflect the assumption that no spread of infection can occur while an infected person is in the incubating stage.

Another variable that can alter the transmission rate and persistence of disease is size of community. Smith (22) summarized data evaluating the link between community size and spread of some infectious diseases and found that the larger the community, the higher the rate of transmission. This observation was found to be true for measles, scarlet fever, diphtheria, and whooping cough (pertussis), but smallpox was not analyzed (22-24). Arita et al. (25) found a correlation between increasing density of smallpox-susceptible persons and the persistence of smallpox within a population but did not estimate the relationship between susceptible population density and transmission rate. Our model allows for the impact of different densities of susceptible persons by adjusting the average transmission rate.

### Numbers Initially Infected and Rate of Transmission

Based on Henderson's comment that an outbreak of smallpox ". . . in which as few as 100 people were infected would quickly tax the resources of any community" (1), we initially assumed that 100 persons would be effectively exposed, infected, and become infectious. We set the average transmission rate at 3, which is notably higher than most historical averages. (A mathematical review of the transmission of smallpox appears in Appendix I, available at URL: http://www.cdc.gov/ncidod/eid/vol7no6/meltzer_appendix1.htm). We define the term "transmission rate" as the number of persons infected per infectious person, rather than the number of persons infected during a standardized unit of time. During sensitivity analyses, we altered both the number of persons initially infected and the rate of transmission.

### Modeling the Effects of Potential Interventions

We examined the effect of quarantine and vaccination, alone and in combination (objective 3). Quarantine was modeled by removing daily a fixed proportion of a cohort of infectious persons, starting on the day that they become overtly symptomatic. For example, we assumed that 50% of all persons with rashes on day 1 of the overtly symptomatic period would be successfully quarantined and not infect anyone else. Fifty percent of those who missed quarantine on day 1 of rash would be quarantined on day 2.[4] This proportionate reduction would continue for the duration of time that persons are likely to infect others. The model also calculated the number of infectious persons needed to be quarantined under a given scenario.

For a vaccination-only strategy to stop transmission, sufficient susceptible persons must be effectively vaccinated so that the number of persons infected per infectious person is less than 1. We thus evaluated how long it would take to stop an outbreak if the level of transmission were reduced to 0.99 persons infected per infectious person. We also calcu-

[3]The United States stopped routine vaccination of the civilian population in 1972 (5). In July 1998 in the United States, there were approximately 109.9 million persons ≤30 years of age, representing 41% of the total resident population (20). Most of these people have not been vaccinated against smallpox. In addition, the immunologic status of those who were vaccinated >30 years ago must be considered. Historical data indicate that vaccination 20 to 30 years ago may not protect against infection but will often protect against death (8,21). No reports, however, define the probability of such persons' transmitting the disease to susceptible persons. Faced with such uncertainty, we chose the simplest approach of assuming an unlimited supply of susceptible persons.

[4]At a 50% daily removal rate, a cohort of all those beginning the first day of overt symptoms is entirely removed in 7 days (8 to 10 days postincubation), with 90% removed in 4 days after they enter the overtly symptomatic period. At a 25% daily removal rate, a cohort is entirely removed 17 days after entering the overtly symptomatic period (18 to 20 days postincubation), with 90% removed in 9 days after entering the overtly symptomatic period. The calculated numbers of those quarantined relate only to those who are infectious (i.e., overtly symptomatic). The model does not take into account those who might also be quarantined along with the infectious persons, such as unvaccinated household contacts and other exposed persons.

lated the smallest vaccine-induced reduction in transmission required to stop the outbreak within 365 days postrelease. This calculation was done by an iterative process in which the rate of transmission was reduced until the number of new cases per day reached approximately zero 365 days after release. To estimate the impact of vaccination, we assumed that a vaccination campaign would immediately reduce the risk of transmission, and we did not model the time required from vaccination to effective vaccine-derived immunity. This assumption may overstate the impact of vaccination, particularly in terms of how quickly a vaccination campaign could stop an outbreak.

Lane and Millar estimated that continuing routine childhood immunization against smallpox in the United States from 1969 to 2000 would cause 210 vaccine-related deaths (26). That calculation was made before the population included substantial numbers of immunocompromised persons (e.g., HIV- or cancer therapy-induced immune suppression). Because of the potential for adverse vaccine-related side effects,[5] it may be prudent to attempt to limit the number of persons vaccinated. We therefore calculated the impact of limiting the numbers vaccinated so that transmission would be reduced by just 25%, from 3 to 2.25 persons infected per infectious person, combined with a daily quarantine rate of 25%. We also calculated, by an iterative process, the smallest vaccine-induced reduction in transmission required to stop the outbreak within 365 days postrelease when combined with a daily quarantine rate of 25%.

### Start of Interventions

We considered the effect of starting large-scale, coordinated interventions on days 25, 30, and 45 postrelease, assuming release on day 1. Twenty-five days assumes 15 days for the first signs of overt symptoms (Figure 2), 2 days for initial clinical diagnosis, 1 day for specimen transport, 3 days for laboratory confirmation, and 4 days to mobilize and begin appropriate large-scale interventions.[6] Although interventions may begin on a small scale earlier than day 25, in the model the term "start date of interventions" refers to the date when a full-scale and comprehensive intervention begins (i.e., the model does not allow for a gradual increase of intensity in interventions). If we assume that an average of 15 days will be needed for those infected to become infectious (Figure 2), 30 days represents the time when the first generation of cases (those infected by the index cases) will begin to show overt symptoms. Forty-five days represents the time needed for the second generation of cases (those infected by the first generation) to show overt symptoms.

### Numbers Vaccinated per Case: Stockpile Issues

To determine the number of persons that must be vaccinated, we searched for reports of successfully contained smallpox outbreaks in which both the number of cases and the number of doses of vaccine administered were recorded. These data allowed us to assemble a data set of doses used per case, which was then fitted to probability distributions by using specialized software (Bestfit, Palisade Corp, Newfield, NY). The probability distribution that gave the "best fit," judged by standard tests (chi square, Kolmogorov-Smirnov, Anderson-Darling), provided the mean and median number of doses historically used per case of smallpox, as well as confidence intervals (e.g., 95th, 90th, and 10th percentiles). We then estimated the total number of vaccine doses that should be stockpiled by multiplying the estimated doses per case by the number of cases estimated by the Markov chain model (objective 4).

### Other Potential Interventions

We did not consider other potential preparations, such as routine mass immunizations against smallpox. Reasons for this exclusion include uncertainties about cost, vaccine safety, duration of vaccine efficacy, and the probability of such an event.

### Sensitivity Analyses

We examined the effect on the number of daily and total cases when the number initially infected was changed from 100 to 1,000 and the transmission rate was decreased to 2 or increased to 5 persons infected per infectious person. We also used the model to determine the minimum level of interventions needed to ensure that transmission stopped by given target dates. We chose 75, 150, and 225 days postrelease as the examples of target dates, representing 5, 10, and 15 generations of smallpox, respectively. The minimum levels of intervention needed to achieve these targets were determined by an iterative process, altering the level of the intervention(s) until the number of new cases per day reached zero on each target date.

## Results

### Effect of Transmission Rate and Numbers Initially Infected

We calculated the hypothetical effect of allowing smallpox to spread without intervention, assuming an unlimited supply of smallpox-susceptible persons. The data demonstrate that the most important mathematical variable is the assumed rate of transmission. For a given number of persons initially infected, doubling the number infected per infectious person causes a massive increase (greater than 2 orders of magnitude) in the cumulative total cases at 365 days (Table 1).

### Effect of Intervention: Quarantine Only

A quarantine-only program can stop an outbreak of smallpox, but it takes a daily removal rate of at least 50% to ensure that disease transmission will cease. At a quarantine rate of 50% starting on day 30 postrelease, the daily number of new cases would peak at approximately 50 cases per day, with no new cases on day 240 and a cumulative total of approximately 2,300 cases (Figure 4). If 50% quarantine began 5 days earlier, on day 25 postrelease, the total cases would be approximately 1,750 and the maximum number of daily new cases would be 20 per day (Figure 4). A 15-day delay in starting quarantine programs, to day 45 postrelease, results in approximately 6,800 total cases and a maximum of almost 120 new cases daily (Figure 4).

[5]The number, severity, and cost of vaccine-induced side effects is the subject for a separate paper.
[6]Allowing 3 days for laboratory confirmation assumes that virus loads in clinical specimens may be insufficient to allow use of rapid assays and confirmation must await the results of a culture-based assay, which takes approximately 72 hours. Rapid laboratory confirmation, within 24 hours, is possible.

Table 1. Estimates of cumulative total smallpox cases after 365 days with no intervention

| No. initially infected[a] | No. infected per infectious person[b] | Cumulative total no. of smallpox cases, days postrelease[c] | | | |
|---|---|---|---|---|---|
| | | 30 days | 90 days | 180 days | 365 days |
| 10 | 1.5 | 31 | 214 | 2,190 | 224 thousand |
| 10 | 3.0 | 64 | 4,478 | 2.2 million | 774 billion |
| 1,000 | 1.5 | 3,094 | 21,372 | 219,006 | 22 million |
| 1,000 | 3.0 | 6,387 | 447,794 | 222 million | 77 trillion |

[a] Number initially infected refers to those who are exposed during a release so that they subsequently become infectious to others. This scenario excludes those who are exposed but either do not become ill (i.e., are immune or are not exposed to an infectious dose) or do not become infectious (residual immunity from prior vaccination may be sufficient to prevent onward transmission).
[b] The number of persons infected per infectious person is the transmission rate.
[c] Assumes an unlimited supply of smallpox-susceptible persons.

### Effect of Intervention: Vaccination Only

A vaccination-only program that reduces the rate of transmission to 0.99 persons infected per infectious person will eventually stop an outbreak, but not within 365 days postrelease, even if it is begun on day 25 postrelease (Figure 5). To stop the outbreak by day 365 postrelease, a vaccination campaign starting on day 30 must reduce transmission to approximately 0.85 persons infected per infectious person (Figure 5), resulting in a cumulative total of 2,857 cases. If the same intervention were started on day 25 postrelease, the cumulative total would decline to 2,125 cases. Delaying the start of the intervention to day 45 postrelease would result in 3 new cases per day and a cumulative total of 8,347 cases on day 365.

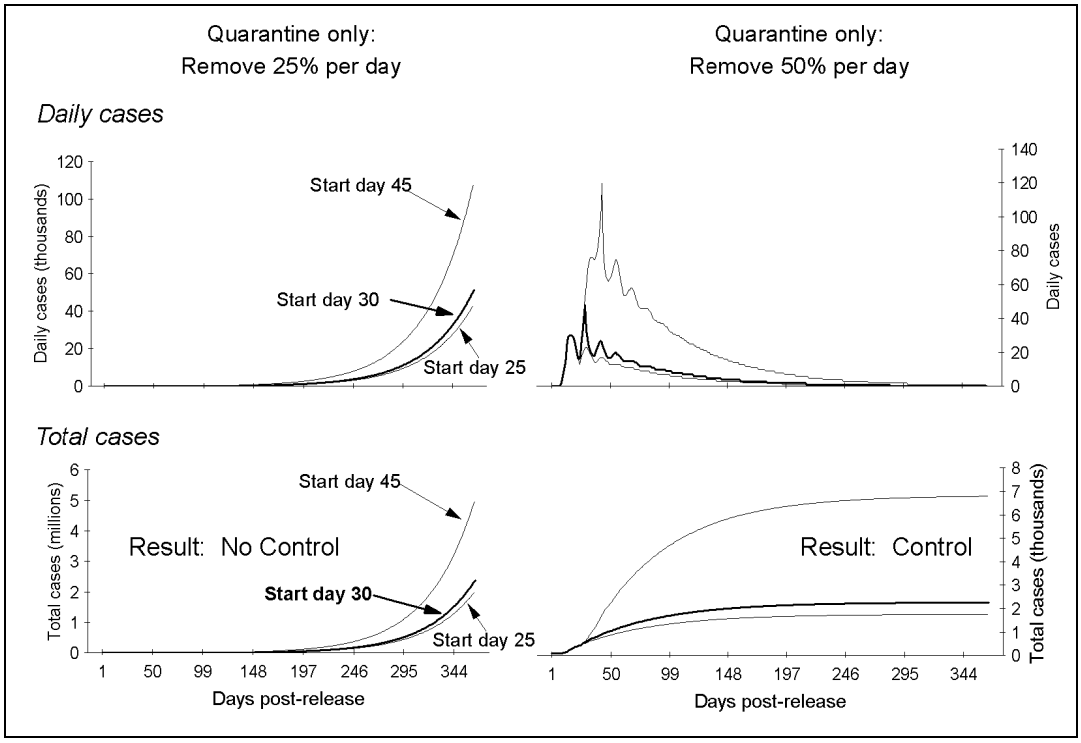


Figure 4. Daily and total cases of smallpox after quarantining infectious persons at two daily rates and three postrelease start dates. The graphs demonstrate that if quarantine is the only intervention used, a daily removal rate of $\geq$ 50% is needed to stop transmission within 365 days postrelease. At a 25% daily removal rate of infectious persons by quarantine, a cohort of all those entering the first day of overt symptoms (i.e., rash) is entirely removed within 17 days (18 to 20 days postincubation) after the first day of overt symptoms, with 90% removed within 9 days. At a 50% daily removal of infectious persons by quarantine, a cohort of all those entering their first day of overt symptoms (i.e., rash) is entirely removed within 7 days (8 to 10 days postincubation) after the first day of overt symptoms, with 90% removed within 4 days. The daily rate of removal (quarantine) relates only to the removal of those who are infectious (i.e., overtly symptomatic). The rate does not include any persons who may be quarantined along with overtly symptomatic patients, such as unvaccinated household contacts. Data generated by assuming 100 persons initially infected and a transmission rate of 3 persons infected per infectious person. For clarity, the graphs of daily cases do not include the assumed 100 initially infected persons. The graphs of total cases include the 100 initially infected

### Effect of Intervention: Quarantine and Vaccination

When combined with a quarantine rate of 25%, to stop transmission by day 365 postrelease, vaccination has to effectively reduce the rate of transmission by at least 33%, from 3 persons infected to 2 persons infected per infectious person (Figure 6). Although transmission will be halted,[7] the total number of cases would be approximately 4,200, which is 82% greater than the total if a 50% daily reduction quarantine-only program is assumed (Figure 4). Starting on day 25 postrelease reduces the total number of cases to approximately 3,200 (Figure 6). Delaying the start of a combined intervention to day 45 postrelease increases the total number of cases to approximately 12,400.

[7] Even by reducing transmission from 3 to 2 persons per infectious person and quarantining infectious persons at a rate of 25% per day, the number of new cases at day 365 is 3, not zero (i.e., transmission is not quite completely stopped) (Figure 6). For transmission to cease completely, vaccination must either achieve a 38% reduction in transmission to 1.85 cases per infectious person (assuming a daily quarantine rate of 25%), or quarantine must achieve a 29% daily reduction in the number of infectious persons (assuming vaccination reduces transmission by 33%).

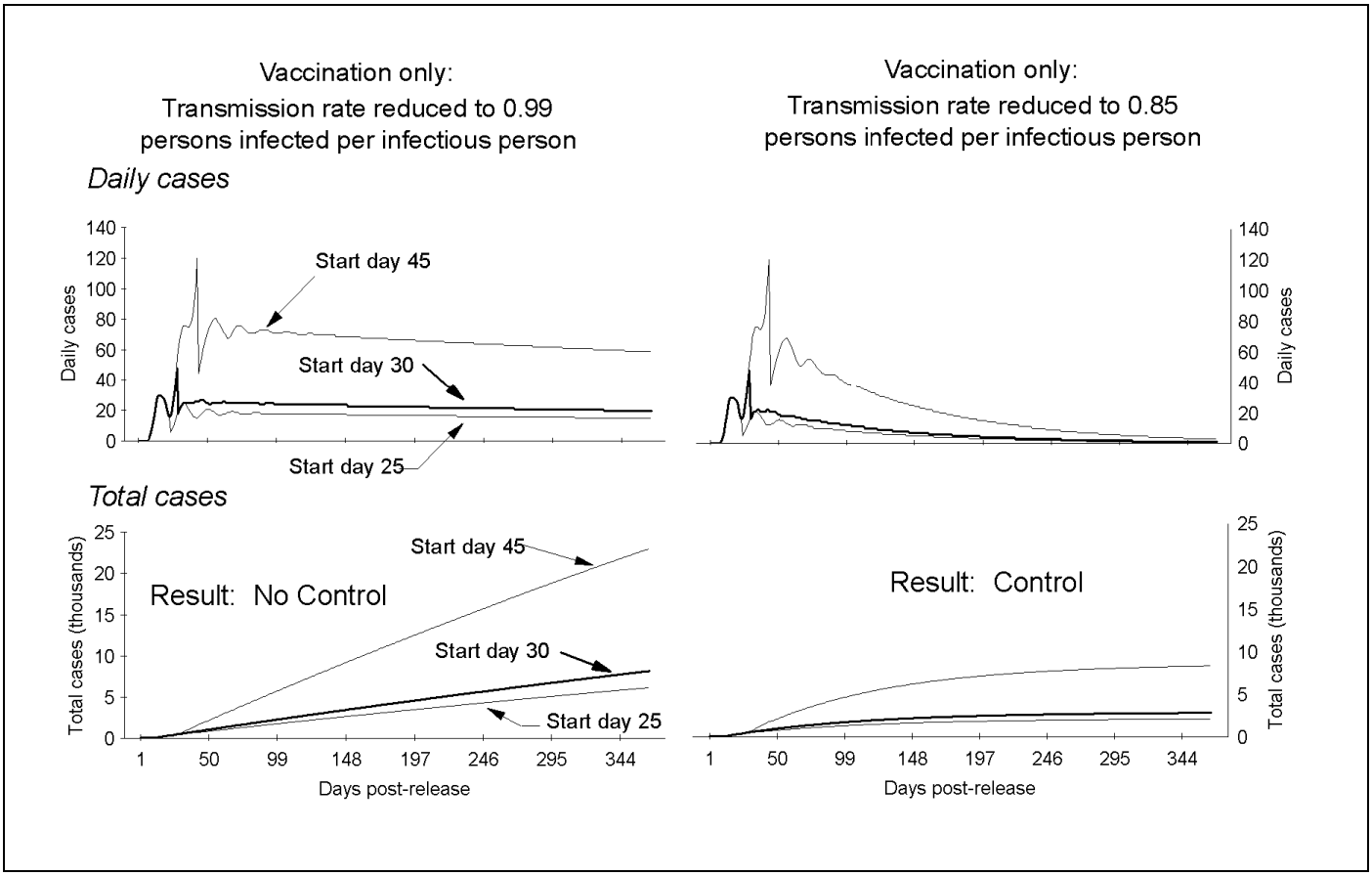


Figure 5. Daily and total cases of smallpox for two vaccine-induced rates of transmission and three postrelease start dates. The graphs show that, while reducing the transmission rate to 0.99 persons infected per infectious person reduces the daily number of cases over the period studied, vaccination must reduce the rate of transmission to 0.85 persons infected per infectious person to stop the outbreak within 365 days postrelease. Data were generated by assuming 100 initially infected persons and an initial transmission rate of 3 persons infected per infectious person. For clarity, the graphs of daily cases do not include the assumed 100 initially infected. The graphs of total cases include those initially infected.

### Effect of Intervention: Number of Infectious Persons Quarantined

With a quarantine-only intervention of 50% daily rate of removal, starting on day 30 postrelease, the peak number of daily removals is 69 infectious persons, occurring on day 30 (start day) with a cumulative total of 2,166 infectious persons quarantined. With a combination of a 33% vaccine-induced reduction in transmission and a 25% daily removal quarantine program, the peak number of daily removals is 34 (start day 30), but the cumulative total that must be quarantined is approximately 3,970 infectious persons.

### Sensitivity Analyses: Effect of Changing Input Values

Reducing the transmission rate to two results in a quarantine-only program with a 25% daily removal rate almost stopping transmission (Table 2). Delaying the start of such an intervention to day 45 but combining it with a vaccination campaign, which reduced transmission by 33%, would halt the outbreak by Day 365 (Table 2). For the same intervention start date, increasing the assumed transmission rate from 2 to 5 persons infected per infectious person does not proportionately increase the cumulative total number of cases at day 365. Even with a quarantine rate of 25% removal per day, assuming that vaccination concurrently reduces transmission by 66%, the cumulative total number of cases on day 365 is 19,821 (Table 2). For any given scenario, increasing the number initially infected from 100 to 1,000 increases both the cumulative totals and the daily number of new cases at day 365 by a factor of 10 (Table 2). Similarly, reducing the number of those initially infected from 100 to 10 would cause a proportionate reduction in both cumulative totals and daily numbers (data not shown; additional results in Appendix II, available at URL: http://www.cdc.gov/ncidod/eid/vol7no6/meltzer_appendix2.htm).

### Sensitivity Analyses: Minimum Levels of Intervention to Achieve Target Days

The earlier the target date for stopping an outbreak, the larger the minimum vaccine-induced reduction in transmission needed to achieve zero transmission (i.e., outbreak stopped). For example, assuming a transmission rate of 3 and a 25% daily removal rate, a target date of day 225 requires a 45.2% vaccine-induced reduction in transmission to 1.65 persons infected per infectious person (Table 3). Reducing the target date to day 75 requires a 76.7% vaccine-induced reduction in transmission to 0.70 persons infected per infectious person (Table 3). Again, delay in starting interventions makes it notably more difficult to stop an outbreak by a given target date. For example, to achieve a target date of day 75 with a 50% daily removal rate, starting interventions on day 45 requires a vaccine-induced reduction in transmission of 81.2%, to 0.57 persons

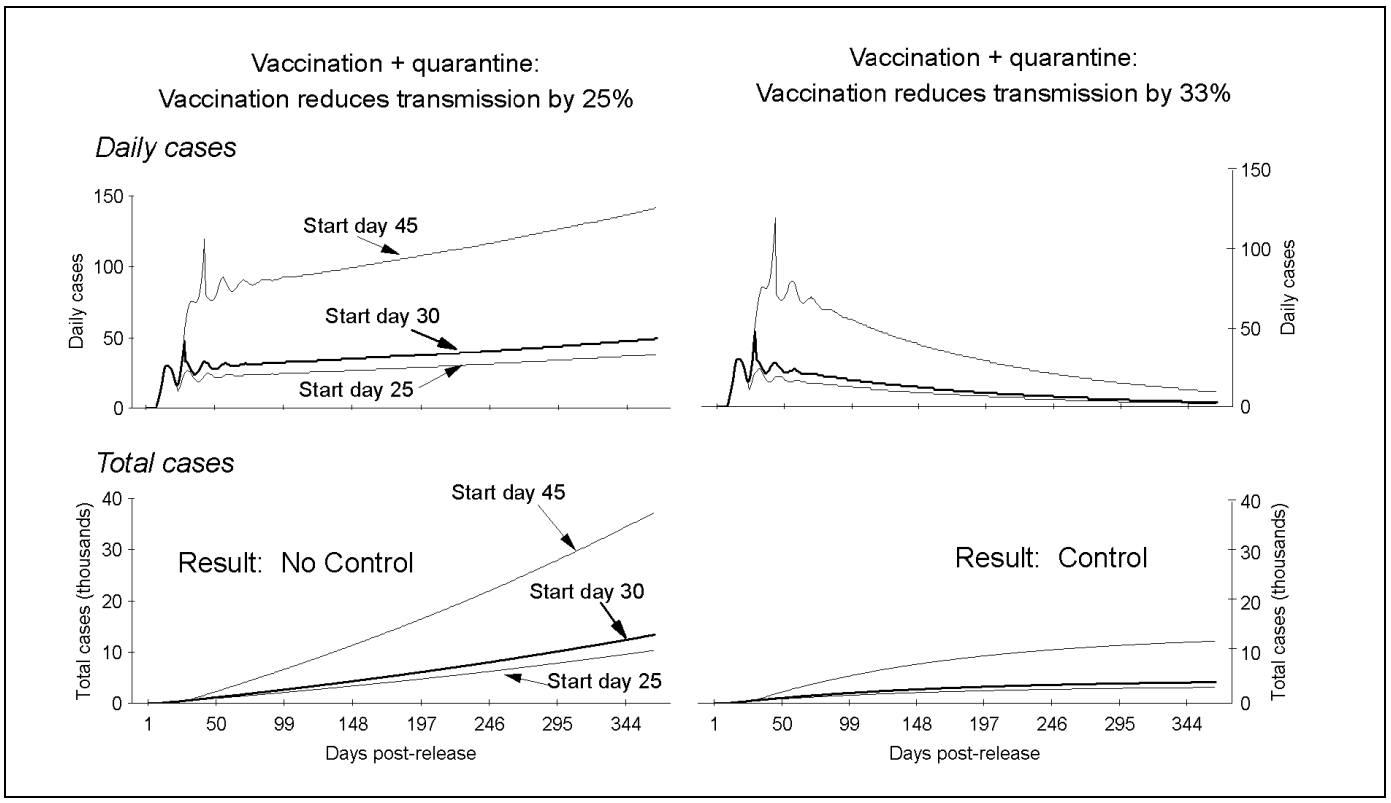


Figure 6. Daily and total cases of smallpox after a combined quarantine (25% daily removal rate) and vaccination campaign for two vaccine-induced reductions in transmission and three postrelease start dates. The graphs show that, when combined with a daily quarantine rate of 25%, vaccination must achieve a ≥33% reduction in transmission to stop the outbreak. At a 25% daily removal rate of infectious persons by quarantine, a cohort of all those entering their first day of overt symptoms (i.e., rash) is entirely removed within 17 days (18 to 20 days after incubation) after the first day of overt symptoms, with 90% removed within 9 days. Removal is assumed to start same day as vaccinations. The daily rate of removal by quarantine relates only to the removal of those who are infectious (i.e., are overtly symptomatic). The rate does not include any persons who may be quarantined along with overtly symptomatic patients, such as unvaccinated household contacts. Vaccinating contacts or potential contacts is assumed to result in 25% and 33% reductions in transmission, so that the transmission rate is reduced from 3 to 2.25 and 2 persons infected per infectious person, respectively. Data were generated by assuming 100 initially infected persons and an initial transmission rate of 3 persons infected per infectious person. For clarity, the graphs of daily cases do not include the assumed 100 initially infected persons. The graphs of total cases include those initially infected.

Table 2. Sensitivity analyses: Effect on number of cases of smallpox due to variations in numbers initially infected, numbers infected per infectious person, intervention start days, and quarantine and vaccination effectiveness[a]

| No. initially infected[b] | No. infected per infectious[c] | Start day[d] | Quarantine: % removal per day[e] | Vaccination: % reduction transmission[f] | Impact: Cumulative total at 365 days | Impact: Daily cases at 365 days | Increase or decrease (+/-[g]) |
|---|---|---|---|---|---|---|---|
| Base:100[h] | 3.0 | 30 | 25 | 33 | 4,421 | 3 | - |
| 100 | 2.0 | 30 | 25 | Nil | 2,455 | 2 | - |
| 100 | 2.0 | 30 | 10 | 25 | 10,512 | 2 | - |
| 100 | 2.0 | 45 | 25 | 33 | 1,548 | 0 | - |
| 100 | 5.0 | 30 | 25 | 66 | 4,116 | 0 | - |
| 100 | 5.0 | 45 | 25 | 66 | 19,821 | 1 | - |
| 1,000 | 2.0 | 30 | 10 | 25 | 105,117 | 511 | + |
| 1,000 | 2.0 | 30 | 10 | 33 | 32,125 | 42 | - |

[a]Table 1, Appendix II (see online) is an expanded version of this table.
[b]Number initially infected refers to those who are exposed during a release such that they become infectious. This excludes those who are exposed but either do not become ill or do not become infectious.
[c]The number of persons infected per infectious person is the transmission rate.
[d]Start day, for both quarantine and vaccination interventions, refers to the day postrelease, with the day of release being day 1.
[e]Quarantine refers to removal of infectious persons only, starting on the first day of overt symptoms (i.e., rash). At a 25% daily removal rate, a cohort of all those entering the first day of overt symptoms is entirely removed in 17 days (18 to 20 days postincubation) after day 1 of overt symptoms, with 90% removed in 9 days. At a 10% daily removal, a cohort of all those entering the first day of overt symptoms is entirely removed in 44 days (45 to 47 days post incubation) after day 1 of overt symptoms, with 90% removed in 22 days.
[f]Vaccination is assumed to reduce the transmission rate by a given percentage (e.g., 25% reduction results in transmission declining from 2.0 to 1.5 persons infected per infectious person, and 33% reduces transmission from 2.0 to 1.32).
[g](+) = an increasing rate of daily cases on day 365, and thus the modeled interventions will not stop the transmission of smallpox. (-) = a decreasing rate of daily cases, such that the interventions modeled will eventually stop the transmission of smallpox.
[h] See Figure 6 for complete results related to the base case in the initial modeling scenario.

infected per infectious person (Table 3). If a 25% quarantine-induced daily removal rate is assumed, then vaccination must reduce transmission by 91.5% to 0.26 persons infected per infectious person (Additional results in Appendix II, available at URL: http://www.cdc.gov/ncidod/eid/vol7no6/meltzer_appendix2. htm).

### Vaccinations per Case: Stockpile Issues

We identified 14 outbreaks in which a range of 9 to 102,857 persons were vaccinated per case of smallpox (Table 4). The mean was 14,411 persons vaccinated per case (median 2,155). When fitted to a Gamma probability distribution (35), the 95th, 90th, and 10th percentiles were 7,001, 4,329, and 3.5 doses per case, respectively (Table 4).

In Yugoslavia the number vaccinated per case was approximately 5 times greater than in any other outbreak considered (31). If the Yugoslavia data are removed from the data set (Table 4), the simple average doses per case would be 6,370 (56% decrease), with a median value of 1,801 (16% decrease) doses per case.

If one assumes 4,200 cases result from 100 index cases and a combined quarantine and vaccination program (start day 30: Figure 6), and one uses a median of 2,155 persons vaccinated per case (Table 4), 9,051,000 doses must be made available for use (4,200 x 2,155). The 95th, 90th, and 5th percentiles of this estimate are 29,404,200, 18,181,800, and 14,700, respectively. When the assumed number of persons infected per infectious person is set at 2, the number of cases declines to 1,548 (start on day 45: Table 2), and 3,335,940 vaccine doses must be made available for use (2,155 x 1,548), with 95th, 90th, and 5th percentiles of 10,837,548, 6,701,292, and 5,418, respectively.

Table 3. Sensitivity analyses: Minimum levels of intervention needed to stop transmission of smallpox by days 75, 150, and 225 postrelease

| Target stop day[a] | Start day of interventions[a] | Numbers infected per infectious person[b] | Quarantine: Minimum % removal per day[c] | Vaccination: Minimum % reduction in transmission[d] |
|---|---|---|---|---|
| 75 | 30 | 2 | 25.0 | 58.0 (0.84) |
| 75 | 30 | 3 | 25.0 | 76.7 (0.70) |
| 75 | 30 | 5 | 50.0 | 78.9 (1.06) |
| 75 | 45 | 3 | 50.0 | 81.2 (0.57) |
| 150 | 30 | 2 | 25.0 | 25.8 (1.49) |
| 150 | 30 | 3 | 25.0 | 53.7 (1.39) |
| 150 | 30 | 5 | 50.0 | 55.7 (2.22) |
| 150 | 45 | 3 | 50.0 | 33.3 (2.00) |
| 225 | 30 | 2 | 25.0 | 14.3 (1.72) |
| 225 | 30 | 3 | 25.0 | 45.2 (1.65) |
| 225 | 30 | 5 | 50.0 | 46.5 (2.68) |
| 225 | 45 | 3 | 50.0 | 14.8 (2.56) |

See Appendix II, Table 2 (online) for an expanded version of this table.
[a]Target stop day and start day of interventions refer to days postrelease, with day of release being day 1.
[b]The number of persons infected per infectious person is the transmission rate.
[c]Quarantine refers to removal of infectious persons only, starting on the first day of overt symptoms (i.e., rash). Rates are the minimum rates needed, when combined with vaccination, to ensure that there is zero transmission by the target date. At a 25% daily removal rate of infectious persons, a cohort of all those entering their first day of overt symptoms is entirely removed in 17 days (18-20 days postincubation) after day 1 of overt symptoms, with 90% removed in 9 days. With 50% daily removal of infectious persons, a cohort of all those entering the first day of overt symptoms is entirely removed in 7 days (8 to 10 days postincubation) after day 1 of overt symptoms, with 90% removed in 4 days.
[d]Vaccination assumed to reduce the transmission rate by a given percentage (e.g., 25% reduction results in transmission declining from 3.0 to 2.25 persons infected per infectious person). Percentages are the minimum percentage reduction in the assumed rate of transmission needed, when combined with quarantine, to ensure zero transmission by the target date. The resultant transmission rate, after reduction, is in parentheses.

## Discussion

The greatest simplification in building our model was the assumption that the supply of susceptible persons was unlimited, so that any specified rate of transmission would be sustained for at least 365 days. In reality, many factors, such as existing immunity and behavior modifications by society (e.g., voluntary or forced quarantine) could limit the supply of susceptible persons, reducing the total number of cases in a 1-year period.

Supply of susceptible persons and assumed rate of transmission are the most important variables influencing the total number of smallpox cases (Tables 1,2). Historically, average transmission rates were well below three persons infected per infectious person (Appendix I, available at URL: http://www.cdc.gov/ncidod/eid/vol7no6/meltzer_appendix1.htm). Variables that can affect the average rate of transmission of smallpox include seasonality, group size, and type of contact ("face-to-face" or "incidental;" Appendix I, Table 5). Our model does not explicitly allow for consideration of such variables, and adjustments to transmission rate resulting from changes in factors such as group size must be done externally to the model.

Another result of assuming an unlimited supply of susceptible persons is that the impact of multiple releases does not "need" to be explicitly modeled. That is, in our model it does not matter if the release initially infects 100 persons who are standing shoulder to shoulder or are each separated by 500 miles. The two variables that can be manipulated to act as proxies for modeling the impact of multiple releases and geographically diverse sites are the transmission rate and the day of the start of interventions. For example, multiple releases may be assumed to result in a lower average transmission rate. Simultaneously, such releases may cause confusion among authorities, the public, and the media, resulting in delay in starting effective interventions. Similarly, releases of smallpox among those perhaps disinclined to interact with authorities (e.g., homeless persons) may go undetected for longer periods of time, also resulting in delayed interventions. We present results from our model of the effect of assuming different transmission rates and start days for an intervention (Tables 1-3). The net result of using these proxy variables to model potential scenarios is that we probably overestimate the spread of disease and the numbers infected. Nonetheless, we feel that the degree of overestimation will probably not substantially affect estimates for thetotalnumberofdosesofvaccinethatshouldbestockpiled.

Another limitation of the model is that it does not explicitly answer the question of how many persons (or what proportion of the population) need to be vaccinated for the transmission rate to decline by, say, 33%. To answer this question, we would need to know two pieces of information:

Table 4. Doses of vaccine used to control outbreaks of smallpox: Numbers vaccinated per confirmed case from a variety of outbreaks, 1961-1973

| Site | Year | Population % susceptible | No. of cases | Total vaccinated | Doses used per case | Source |
|---|---|---|---|---|---|---|
| Saiwara village, India | 1968 | 8 | 40 | 1,358[a] | 34 | 27 |
| Nathawala village, India | 1969 | 12 | 12 | 450[b] | 38 | 27 |
| Bawku, Ghana | 1967 | n/a | 66 | 165,449 | 2,507 | 28 |
| Rural Afghanistan | 1969 | n/a[c] | 6 | 508[d] | 85 | 29 |
| Nuatja subdivision, Togo | 1969 | n/a | 6 | 10,818 | 1,803 | 30 |
| Anéono subdivision, Togo | 1969 | 40 | 47 | 294,274 | 6,261 | 30 |
| Yugoslavia | 1972 | n/a | 175 | 18 million | 102,857 | 31 |
| Utinga City, Brazil | 1969 | 57 | 246 | 2,188 | 9 | 32 |
| Botswana | 1973 | 17-27[e] | 30 | 50,000 | 1,667 | 33 |
| London, UK | 1961 | n/a | 3 | 62,000 | 20,667 | 34 |
| West Bromwich, UK | 1961 | n/a | 2 | "limited"[f] | n/a | 34 |
| Bradford, UK | 1961 | n/a | 14 | 250,000 | 17,857 | 34 |
| Birmingham, UK | 1962 | n/a | 1 | "limited"[f] | n/a | 34 |
| Cardiff, UK | 1962 | n/a | 47 | 900,000 | 19,148 | 34 |
| | | | | Mean | 14,411 | |
| | | | | Median | 2,155 | |
| | | | | 95th perct.[g] | 7,001 | |
| | | | | 90th perct.[g] | 4,329 | |
| | | | | 10th perct.[g] | 3.5 | |

[a]This population includes 1,069 revaccinations, accounting for 79% of total vaccinations.
[b]This population includes 323 revaccinations, accounting for 72% of total vaccinations.
[c]The source did not provide population-based estimates of preoutbreak vaccination coverage (as determined by a vaccine scar survey). However, in the four households that contained the six cases, of the 18 family members present at the time of the investigation, 6 (33%) had evidence of preoutbreak vaccination or variolation.
[d]This number excludes some children who had been vaccinated 15 days before the outbreak investigation.
[e]In the sample (n=68,065), susceptibility varied by age. Smallpox vaccination scars were noted among 76% of those $\leq$5 years of age, 83% of those 6 to 14 years of age, and 79% of those $\geq$15 years of age.
[f]The health authorities for West Midlands, which dealt with two of the importations (West Bromwich, Birmingham, UK), limited vaccinations to "...established contacts and medical and ancillary staffs placed at definite risk..." (34). Thus, although the source provides no estimates of the number vaccinated, the description of those targeted for vaccination can lead to the hypothesis that <1,000 persons were vaccinated per case.
[g]The percentiles were calculated by fitting the data to a Gamma distribution (values of parameters: $\alpha = 0.25$; $\beta = 58,400$). The chi-square value of the fit of the data to the distribution was 20.57 ($p>0.01$), the Kolmogorov-Smirnov test value was 0.1262 ($p>0.15$), and the Anderson-Darling test statistic was 0.3147 ($p>0.15$).

first, what percentage of the population is truly susceptible to smallpox and could become infectious to others; and second, how would these susceptible persons interact with those infected?[8]

### Vaccination Alone or Combined with Quarantine?

The results from the model demonstrate that it is theoretically possible to completely halt the spread of smallpox by quarantine only (Figure 4; Tables 2,3). The level of quarantine needed, however, may prove impossible to enforce. On the other hand, historically, mass vaccinations alone did not always stop the transmission of smallpox (7,8). Thus, relying solely on either intervention would appear to be unwise, so that a combination of vaccination and quarantine should beused.

Using quarantine has the benefit of lowering the level of effective vaccination needed to stop transmission (Tables 2,3). Furthermore, compared with a vaccination-only intervention, a combined quarantine and vaccination campaign will produce fewer total cases and stop transmission sooner (Table 3). Depending on how vaccination is done, requiring a lower level of effective vaccination could result in fewer vaccinations being administered. Given that the smallpox vaccine occasionally has adverse effects, including death (7,8), any method that reduces the number of vaccinations needed to halt transmission should be examined for possible inclusion into a response plan.

### Doses To Be Stockpiled

The number of estimated doses that must be stockpiled ranges from the 5th percentile estimate of approximately 5,000 doses (assuming approximately 1,500 cases) to a 95th percentile of almost 30 million (assuming approximately 4,200 cases). The latter estimate was generated by assuming an average rate of transmission of three persons infected per infectious person. This assumed level of transmission is well above historical average rates of transmission (Appendix I, available at URL: http://www.cdc.gov/ncidod/eid/vol7no6/meltzer_appendix1.htm). Thus, allowing for factors such as vaccine wastage, stockpiling 40 million doses as recommended by Henderson et al. (5) should be adequate.

[8]Although there are some historical data regarding how infected persons interacted and infected others, all such data were collected when circumstances differed from those of today's societies, particularly with regard to travel and spread of information. Although air and other modes of mass travel were common before smallpox was eradicated, the numbers of travelers and the total miles traveled have vastly increased in the past 30 years. Similarly, although mass media were well known and used in the 1960s and 1970s, more outlets are available to spread information than ever before. It is unknown how these and other changes could affect the spread of smallpox.

Because the pool of smallpox-susceptible persons is now very large, the rate of transmission may be much higher than historical averages, resulting in more cases of smallpox and the need for more vaccine doses stockpiled. For example, if a transmission rate of 5 is assumed and large-scale interventions are started on day 45 postrelease, the 95th percentile of doses that should be stockpiled is 140 million doses (mean 43 million doses; Tables 2,4). Similar estimates are obtained if it is assumed that 1,000 persons are initially infected (Tables 2, 4). Further supporting the argument for stockpiling >40 million doses is the idea that there would be enormous public demand for vaccination in the event of an outbreak.

Stockpiling a large number of doses of smallpox vaccine has three major problems. Building a stockpile of 140 million doses might leave public health officials without needed resources to prepare for and implement other interventions, such as quarantine and public education. Second, a large stockpile poses the problem of deciding how to use it. Investing in such a resource may invite the conclusion that the only suitable response to a deliberate release of smallpox would be a mass vaccination campaign, using as much of the stockpile as possible. An enormous logistical problem would be associated with rapidly vaccinating 140 million persons. Assuming 10 minutes per person vaccinated (excluding patient waiting time), 23 million person-hours would be required to vaccinate 140 million people. In 1947 in New York City it took approximately 1 week to vaccinate 6 million people in response to an outbreak with eight cases (1). An additional problem with trying to mass-immunize >100 million people is that, if a transmission rate of 5 is assumed, disease spread might be so rapid as to "outrun" any mass vaccination attempt (Tables 1,2). The third problem associated with a large stockpile of smallpox vaccine is that a large number of side effects would be generated, including need for treatment with vaccinia immunoglobulin and deaths as a result of adverse reactions (26). Between the demands of vaccination and treatment of side effects, the health-care system would be overburdened, to the detriment of treatment for any other disease or medical emergency.

## Policy Implications

The four most important policy implications from the model results are 1) Delay in intervention will be costly, dramatically increasing the total number of cases; 2) Postrelease intervention should be a combination of quarantine and vaccination; 3) Planning requires not only an appreciation of how many persons may be infected initially, but also an understanding of the likely rate of transmission; and 4) a stockpile of approximately 40 million doses of vaccine should be adequate.

Beyond stockpiling, adequate planning, preparation, and practice must be carried out (36). Such preparation must include training health-care workers to recognize a case of smallpox and what to do if a case is diagnosed. Public health authorities and policymakers need to make detailed plans that fully describe how persons will be quarantined and how quarantine will be enforced. The successful enforcement of quarantine requires political will, public acceptance, and group discipline. Thus, a large part of the preparation for a public health response to smallpox as a bioterrorist weapon must involve educating policymakers and the public as to why quarantine is needed and why relying solely on mass immunizations may not be the magic bullet that some might hope.

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# *Hepatitis E Virus* Sequences in Swine Related to Sequences in Humans, the Netherlands

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*Hepatitis E virus* (HEV), a major cause of viral hepatitis in much of the developing world, has recently been detected in swine in North America and Asia, raising concern about potential for zoonotic transmission. To investigate if HEV is commonly present in swine in the Netherlands, pooled stool samples from 115 swine farms and nine individual pigs with diarrhea were assayed by reverse transcription-polymerase chain reaction (RT-PCR) amplification. HEV RNA was detected by RT-PCR and hybridization in 25 (22%) of the pooled specimens, but in none of the individual samples. RT-PCR amplification products of open reading frames 1 and 2 were sequenced, and the results were compared with published sequences of HEV genotypes from humans and swine. HEV strains from swine in the Netherlands were clustered in at least two groups, together with European and American isolates from swine and humans. Our data show that HEV in swine in the Netherlands are genetically closely related to HEVs isolates from humans. Although zoonotic transmission has not been proven, these findings suggest that swine may be reservoir hosts of HEV.

*Hepatitis E virus* (HEV) is a nonenveloped RNA (7.5-kb) virus, previously classified as a calicivirus but provisionally classified in a separate family of HEV-like viruses (1). HEV is responsible for large epidemics of acute hepatitis and sporadic cases in southeast and central Asia, the Middle East, parts of Africa, and Mexico. Few HEV infections have been reported in nontravelers in industrialized countries, including the Netherlands (2). HEV infection spreads by the fecal-oral route, usually through contaminated water. The clinical illness resembles other forms of acute viral hepatitis, with onset after an 1- to 8-week incubation period. Clinical attack rates are the highest among young adults. In younger age groups, infections are more often anicteric and asymptomatic. Chronic HEV infection has not been observed. Although the death rate is usually low (0.07% to 0.6%), the illness may be particularly severe among pregnant women, with death rates as high as 25% (3). To date, no specific treatment is available for HEV infection. Ensuring a clean drinking water supply remains the best preventive strategy.

Viral excretion begins approximately 1 week before onset of illness and persists for nearly 2 weeks; viremia can be detected during the late phase of the incubation period and in the acute phase of illness (3,4). Long-term persistence of HEV in the body fluids of infected persons seems to be an unlikely reservoir for transmission of HEV (3). Experimental HEV infection in swine has been reported (5), and serologic evidence for HEV infection in swine from areas endemic for human HEV has also been reported (6). Recent isolation of a swine virus resembling human HEV suggests the possibility of zoonotic HEV infection (7).

The objective of this study was to investigate if HEV is prevalent in swine in the Netherlands and to determine the relationship between the strains detected in pigs and those described in humans.

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## Methods

### Fecal Specimens

Stool specimens from swine were collected as part of ongoing surveillance for potential zoonotic microorganisms associated with gastroenteritis in humans (8). From October 10, 1998, through April 21, 1999, fecal samples were collected from 115 pig farms located throughout the Netherlands. Pig samples were collected from fattening pigs 3 to 9 months of age; farm sizes ranged from 22 to 1,600 animals. Individual stool samples were collected from nine pigs with diarrhea.

### Sampling

The sampling strategy was designed to allow monitoring for the presence of pathogens in a large number of animals; it allows detection of microorganisms at the farm level with a prevalence of 5% and 95% confidence (8). Pig farm samples were collected from animals housed in one randomly chosen farm building. A minimum of 20 and a maximum of 60 fresh stool specimens were collected per farm and pooled samples were designated as the farm sample. Fecal samples were stored until testing at -70°C in 15 g/L of Trypton Soya broth (Oxoid CM 129) and 10% glycerol.

### Molecular Detection of HEV

For extraction of viral RNA, stool samples were resuspended in Hanks balanced salt solution (Gibco BRL, Breda, the Netherlands) to a final concentration of approximately 10%. These suspensions were centrifuged at 3,000 x *g* for 20 minutes, and 100 µL was used for RNA extraction. Viral RNA was extracted by binding to size-fractionated silica beads (Sigma, Roosendaal, the Netherlands) in the presence of guanidinium isothiocyanate (GuSCN). Bound RNA was washed and eluted as described (9).

To reduce risk of contamination, one water sample for every four stool specimens was included as a negative control, treated the same way as the fecal samples. For positive controls, three HEV-positive samples (10% fecal suspensions) were used. One human (US-2) and one swine (Meng isolate) HEV-positive sample, both isolated in the United States, were included. The human isolate (US-2) was passaged once in a Cynomolgus macaque and once in a Rhesus monkey (*Macaca mulatta*). The swine isolate was passaged once in a Rhesus monkey. The third positive control sample was a Burmese HEV swine isolate (10), which was passaged once in Cynomolgus monkeys (*M. fascicularis*). Extraction, preparation of master mixes and reactions, and analysis of polymerase chain reaction (PCR) products were done in different rooms with designated sets of pipettes. To avoid false-positive PCR results, the precautions described by Kwok and Higuchi (11) were strictly followed.

We used single-round and nested reverse transcription (RT)-PCR assays with primer pairs, as described by Meng et al. (7), Wang et al. (12), and Schlauder et al. (13). Two of these primer pairs target a section of the open reading frame (ORF)1 gene coding for nonstructural proteins (Table 1). Three primer pairs target the ORF2 part of the HEV genome that codes for the viral structural proteins (Table 1). Primers ORF2-s1 and ORF2-a1 were used for screening and detecting HEV RNA in all fecal samples. This single-round RT-PCR amplifies 197 nucleotides of ORF2. Primer sets ORF1-s1/ORF1-a1 with ORF1-s2/ORF1-a2 and 3156-EF/3157-ER with 3158-EF/3159-IRS were used for nested PCR amplification of specific parts of the ORF1 and ORF 2 encoding regions. Second-round internal primers amplify the 286 and 348 nucleotides of ORF1 and ORF2, respectively.

For RT, 5 µL of RNA was mixed with 4 µL of 45 pmol antisense primer (ORF1a1 for ORF1 and 3157ER for ORF2). The solution was heated to 95°C for 2 minutes, and after cooling on ice, 6 µL of RT buffer was added. The RT reaction was performed in a final volume of 15 µL consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 1 mM each of deoxynucleoside triphosphates (dNTPs), and 5 U of *avian myeloblastoma virus*-RT (Boehringer Mannheim, Almere, Netherlands). The mixture was incubated for 1 hour at 42°C, heated for 5 minutes at 95°C to denature the enzyme, and then placed on ice. Five microliters of the RT mixture was added to the PCR mix, which contained 10 mM Tris-HCl (pH 9.2), 75 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 units AmpliTaq (Perkin Elmer, Nieuwerkerk a/d IJssel, Netherlands), and 15 pmol sense primer (ORF1-s1 for ORF1 and 3156 EF for ORF2). The final volume of the PCR reaction was 50 µL. Mineral oil was added, and 40 amplification cycles (1 minute at 94°C, 1 minute 30 seconds at 55°C, and 1 minute at 74°C each) were performed. The amplification products were analyzed by 2% agarose gel electrophoresis and visualized with UV after ethidium bromide staining. Methods for both amplification rounds of the nested PCR were the same as for the single-round PCR. To measure HEV concentrations in the pooled fecal pig farm samples, end-point dilution PCR was performed with the US-2 sample as a reference.

### Southern Blot Hybridization

RT-PCR was followed by Southern blot hybridizations. An HEV-specific probe was developed based on the consensus sequence of RT-PCR products of the human and swine HEV control samples. The probe sequence was 5'gagaatgcdcagcaggayaaggg3'. For Southern blotting, the RT-PCR products in the agarose gel were denatured by incubating in 0.5 M NaOH for 30 minutes and transferred to a positively charged nylon membrane (Boehringer, Almere, Netherlands) by vacuum blotting (Millipore, Etten-Leur, Netherlands).

Hybridization of HEV RT-PCR products was performed as described for *Norwalk-like virus* by Vinjé et al. (14). Briefly, the nylon membranes were prehybridized for 30 minutes at 42°C in 20 mL 2x SSPE (300 mM NaCl, 20 mM $NaH_2PO_4H_2O$, 2 mM EDTA, pH 7.4) with 0.1% sodium dodecyl sulfate (SDS). The membranes were left for 45 minutes at 42°C to allow hybridization, after addition of 40 pmol of each of the 5'-biotinylated probes (14). The membranes were washed three times for 10 minutes at 42°C with 2x SSPE and 0.1% SDS. Then the membranes were incubated

Table 1. Oligonucleotide primers employed for RT-PCR amplification

| Primer | Sense | Sequence (5' to 3') | Position in genome | Reference |
|---|---|---|---|---|
| ORF1-s1 | Sense | Ctggcatyactactgcyattgagc | 56-79 | 13 |
| ORF1-a1 | Antisense | Ccatcrarrcagtaagtgcggtc | 451-473 | 13 |
| ORF1-s2 | Sense | Ctgccytkgcgaatgctgtgg | 104-124 | 12 |
| ORF1-a2 | Antisense | Ggcagwrtaccarcgctgaacatc | 367-389 | 12 |
| ORF2-s1 | Sense | Gacagaattratttcgtcggctgg | 6298-6321 | 13 |
| ORF2-a1 | Antisense | Cttgttcrtgytggttrtcataatc | 6470-6494 | 13 |
| 3156-EF | Sense | Aaytatgcmcagtaccgggttg | 5687-5708 | 7 |
| 3157-ER | Antisense | Cccttatcctgctgagcattctc | 6395-6417 | 7 |
| 3158-EF | Sense | Gtyatgytyygcatacatggct | 5972-5993 | 7 |
| 3159-IRS | Antisense | Agccgacgaaatyaattctgtc | 6298-6319 | 7 |

[a]Nucleotide positions are numbered according to the Burmese strain (10).

with 1:4,000 diluted streptavidin-peroxidase conjugate (Boehringer, Almere, the Netherlands) for 45 minutes at 42°C in 10 mL of 2x SSPE and 0.5% SDS. After washing three times (10 minutes each) with decreasing concentrations of SDS (0.5%, 0.1%, and 0%) in 2x SSPE, the membranes were incubated for 2 minutes with the enhanced chemoluminescence (ECL) detection reagents (Amersham Life Science, s'Hertogenbosch, the Netherlands), and then were exposed to an ECL hyperfilm (Amersham Life Science) for 30 minutes and overnight to visualize the bound probe.

### Cloning, Sequence Comparison, and Phylogenetic Analysis

HEV RT-PCR products of expected sizes from the pig farm samples were excised from a 2% agarose gel, purified with a Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and cloned into pGEM T-Easy Vector System II (Promega, Madison, WI). After transformation, five positive colonies of each ligation were selected. The pGEM T-Easy Vector was checked for correct insertion size by direct PCR amplification with M13 forward and M13 reverse primers. Correct PCR products were purified with PCR purification kit (Qiagen) and sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Foster City, CA) by the use of PCR primers. Nucleotide sequences were edited by using Seq Ed (V1.03, Applied Biosystems) and aligned by Bionumerics (V2.0 Applied Maths, Kortrijk, Belgium). Distance calculations were done by the Jukes and Cantor correction for evolutionary rate (15). The confidence values of the internal nodes were calculated by performing 100 bootstrap analyses. Evolutionary trees for nucleotide sequences were drawn by the Jukes and Cantor method, with HEV strain Burma (GenBank accession number M73218) bp 125-366 and bp 5,994-6,297 as reference.

### Electron Microscopy

Electron microscopy procedures were performed as recommended by Flewett (16) and Doane and Anderson (17). Briefly, a 10% fecal suspension in phosphate-buffered saline was clarified by centrifugation for 30 minutes at 3,000 x *g* at 4°C. The supernatant fluid was collected and centrifuged for 1 hour at 90,000 x *g* at 4°C. The pellet was resuspended in 1 drop of distilled water, and the grids were negatively stained with 2% K-phosphotungstic acid (pH 7.0). Grids were investigated for the presence of viruses with an electron microscope, model Philips 400T (Philips, Eindhoven, the Netherlands) at 80 kV. Identification of virus particles was based on morphologic criteria, i.e., size and characteristic surface morphology (18). The diameters of the virus particles were measured directly on the negatives, instead of on the prints. A 10x measuring magnifier with metric scale was used for particle measurements. Magnification calibration was performed each year with a crossed-line grating replica. All fecal swine farm samples (n = 115) were screened by electron microscopy for viruses.

## Results

### RNA Detection and Virus Detection

In 20 of the pooled samples from the swine farms, HEV was detected by single-round RT-PCR with the primer pair ORF2-s1/ORF2-a1. None of the nine individual samples from pigs with diarrhea contained HEV RNA by RT-PCR. Southern blot hybridization with a probe designed for both human and swine HEV strains confirmed all RT-PCR-positive reactions and identified 5 more positive pooled samples, for a total of 25 (22%) of 115 HEV-positive farm samples.

Nested RT-PCR of ORF1 and ORF2 fragments, with different primer sets, was performed for sequencing. Nested RT-PCR of the 25 HEV-positive samples with the primer sets targeting ORF1 resulted in PCR products of specific size in 18 samples. Nested RT-PCR with the primer sets targeting ORF2 resulted in PCR products of specific size in 17 samples. Five of these samples were positive in the first amplification round. In one sample, our single-round screening RT-PCR was negative, but the nested RT-PCR was positive.

PCR titers (endpoint dilution PCR) of the pooled fecal pig farm samples were between 10 E.4 and 10 E.2. In comparison, the US-2 isolate had an infectivity titer of approximately 10 E.6 and reached 10 E.2 positive dilutions by PCR (Figure 1).

Electron microscopy analysis of the 25 RT-PCR positive samples revealed particles with HEV-like morphologic features in only one pig farm sample. The diameter of these particles was 31.5 nm.

### Cloning, Sequence Comparison, and Phylogenetic Analysis

For 14 HEV isolates, nucleotide sequences of both ORF1 and ORF2 PCR products were obtained. Cloned sequences from the same sample showed little or no diversity in ORF1 as well as ORF2 fragments. Only cloned sequences were used in the phylogenetic analyses. The sequences reported in this paper have been deposited in GenBank (accession numbers AF336290-336299 and AF335998-336014). Comparison of the nucleotide sequences showed percent nucleic acid identities of 82.0% to 95.5% in the 242-bp fragment of ORF1 and 79.5% to 92.7% in the 304-bp fragment of ORF2 among swine HEV isolates from the Netherlands. The comparative analysis of sequences of the capsid encoding region ORF2 from GenBank indicated that parts of the Dutch swine sequences (NLSW22 and NLSW122) were closely related (90.0% to 90.9%) to the U.S. human and swine strains, and

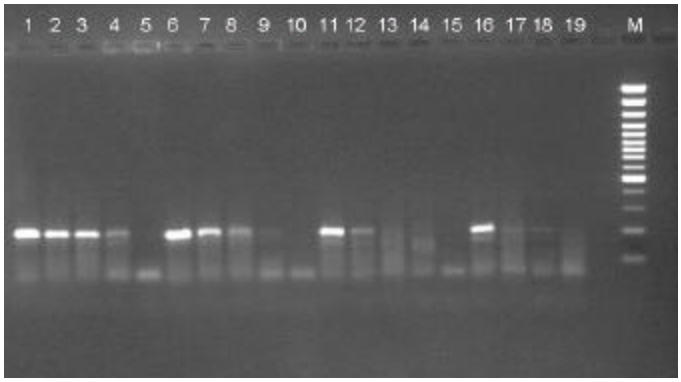



Figure 1. Endpoint dilution polymerase chain reaction with the US2 human *Hepatitis E virus* (HEV) sample as reference (infectivity titer approximately 10 E.6). M: molecular mass marker; lanes 1-4 NLSW50 $10^{-1}$,$10^{-2}$,$10^{-3}$ ;lanes 6-9: NLSW15 $10^{-1}$,$10^{-2}$,$10^{-3}$; lanes 11-14: NLSW20 $10^{-1}$,$10^{-2}$,$10^{-3}$; lanes 16-19 US2 $10^{-1}$,$10^{-2}$,$10^{-3}$. Lanes 5,10 and 15 negative control water samples.

others (NLSW50) were closely related (91.8% to 93.1%) to human and swine strains from Spain (Table 2). Comparison with other isolates from endemic areas showed a nucleotide identity <79.8% in both fragments. Many of these changes did not result in differences at the amino acid level.

By phylogenetic analysis, the swine HEV sequences of ORF1 and ORF2 formed at least two separate clusters. Seven of 14 Dutch isolates were closely related to the U.S. human and swine isolates. The other seven Dutch isolates were closely related to European HEV isolates from humans and swine (Figures 2 and 3).

## Discussion

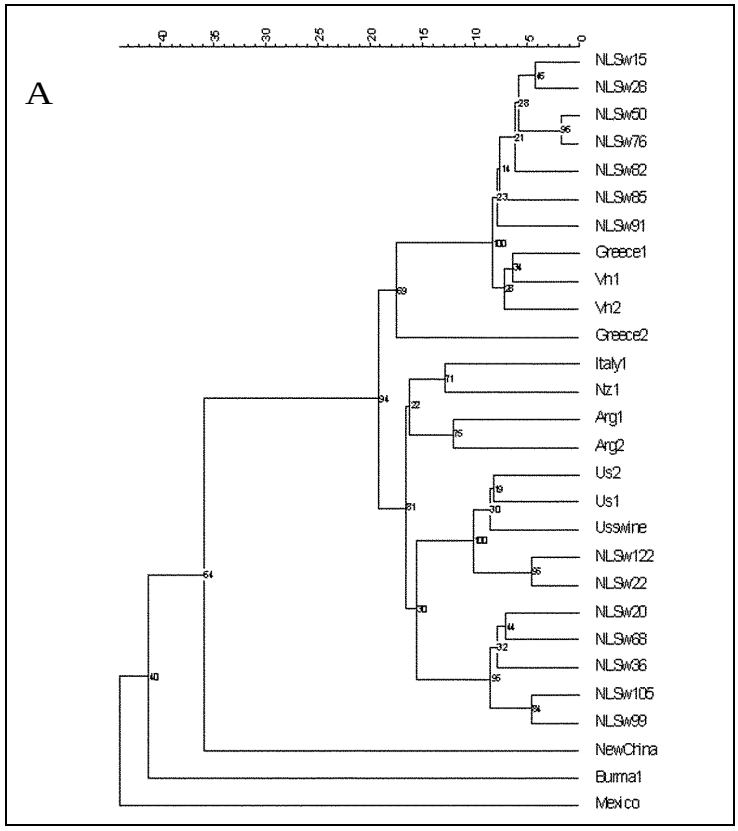
To determine whether HEV is prevalent in swine in the Netherlands, we used RT-PCR methods, with primers located in the HEV ORFs 1 and 2. Cloned sequences of the PCR product showed little or no diversity, suggesting that only one or a few HEV strains circulated in a pig farm. Despite the fact that PCR titers revealed reasonable quantities of virus (Figure 1), particles with HEV-like morphologic features could be detected by electron microscopy in only one of the 25 RT-PCR positive samples. This relatively low number of positives by electron microscopy can be explained by the greater sensitivity of RT-PCR and may also have resulted from freeze-thawing the samples. HEV-like caliciviridae have been described as sensitive to freeze-thawing (27).

This is the first report with direct evidence of HEV in swine in Europe. Pina et al. (21) reported detection of HEV sequences in sewage from a swine slaughterhouse, suggesting that HEV might be present in swine. The rather high prevalence of HEV in commercial swine farms suggests that it is widespread in the general swine population. In this study, clinical symptoms in swine were not recorded, and no overt clinical symptoms were observed. Therefore, a clinical association with HEV infection could not be demonstrated.

Table 2. Nucleotide identity (%) between NLSW *Hepatitis E virus* (HEV) isolates, with respect to other HEV strains, in sections of 242 bases of ORF1 and 304 bases of ORF2[a]

| | ORF1 fragment | | | | ORF2 fragment | | | |
|---|---|---|---|---|---|---|---|---|
| HEV strain | NLSW15 | NLSW22 | NLSW50 | NLSW105 | NLSW15 | NLSW22 | NLSW50 | NLSW105 |
| NZ1 | 82.0 | 85.7 | 82.3 | 84.2 | Na[b] | Na | Na | na |
| Italy | 80.8 | 82.3 | 80.8 | 83.8 | Na | Na | Na | na |
| Arg1 | 82.7 | 85.7 | 83.5 | 88.4 | Na | Na | Na | na |
| Arg2 | 79.5 | 83.3 | 81.0 | 84.8 | Na | Na | Na | na |
| US1 | 80.5 | 92.9 | 82.7 | 87.2 | 86.7 | 90.9 | 83.9 | 86.4 |
| US2 | 80.8 | 91.4 | 83.1 | 85.7 | 85.7 | 90.9 | 83.6 | 86.7 |
| USswine | 81.2 | 87.6 | 83.5 | 84.2 | 86.1 | 90.0 | 83.0 | 85.7 |
| NLSW15 | 100.0 | 82.0 | 94.7 | 83.8 | 100.0 | 83.8 | 90.9 | 82.7 |
| NLSW22 | 82.0 | 100.0 | 84.2 | 87.6 | 83.8 | 100.0 | 83.3 | 85.7 |
| NLSW50 | 94.7 | 84.2 | 100.0 | 85.7 | 90.9 | 83.3 | 100.0 | 84.4 |
| NLSW105 | 83.8 | 87.6 | 85.7 | 100.0 | 82.7 | 85.7 | 84.4 | 100.0 |
| NLSW36 | 84.2 | 86.1 | 85.3 | 91.0 | 83.0 | 84.7 | 83.2 | 89.4 |
| NLSW82 | 93.2 | 85.3 | 93.2 | 83.8 | 91.0 | 79.5 | 91.2 | 83.3 |
| NLSW85 | 91.7 | 85.3 | 93.6 | 86.1 | 91.3 | 92.5 | 92.7 | 84.2 |
| NLSW122 | 82.7 | 95.5 | 83.5 | 88.4 | 83.2 | 82.6 | 83.8 | 86.0 |
| Greece1 | 92.1 | 82.3 | 92.9 | 83.8 | Na | Na | Na | Na |
| Greece2 | 85.7 | 80.8 | 83.1 | 79.3 | Na | Na | Na | Na |
| Egypt | Na | Na | Na | Na | 75.6 | 76.5 | 76.5 | 76.5 |
| Morocco | Na | Na | Na | Na | 77.5 | 77.2 | 78.1 | 76.5 |
| VH1 | 92.1 | 83.5 | 92.1 | 83.8 | 91.1 | 80.9 | 93.1 | 82.9 |
| VH2 | 91.0 | 83.1 | 91.7 | 83.5 | 92.4 | 80.3 | 92.4 | 81.6 |
| E11 | Na | Na | Na | Na | 92.8 | 80.9 | 91.8 | 82.6 |
| Barcelona | 79.3 | 73.7 | 78.2 | 75.9 | 76.9 | 76.2 | 77.8 | 79.0 |
| Nepal | 79.3 | 73.7 | 78.2 | 75.9 | 78.1 | 78.1 | 79.6 | 79.3 |
| Burma1 | 79.7 | 72.9 | 78.6 | 76.3 | 76.9 | 76.8 | 78.4 | 78.1 |
| India1 | 79.0 | 73.7 | 78.6 | 75.6 | 78.7 | 76.5 | 79.0 | 78.7 |
| China1 | 79.0 | 72.9 | 78.6 | 75.6 | 77.5 | 77.8 | 78.4 | 78.4 |
| Pakistan | 79.3 | 73.3 | 78.2 | 75.9 | 77.5 | 77.2 | 78.4 | 77.5 |
| New China(T1) | 80.5 | 77.8 | 79.7 | 78.6 | 79.0 | 79.6 | 78.7 | 77.2 |
| Mexico | 78.2 | 77.8 | 77.5 | 76.3 | 76.0 | 75.4 | 76.0 | 75.1 |

[a]For explanation of isolates' acronyms, see Figure 2 legends.
[b]na= not available

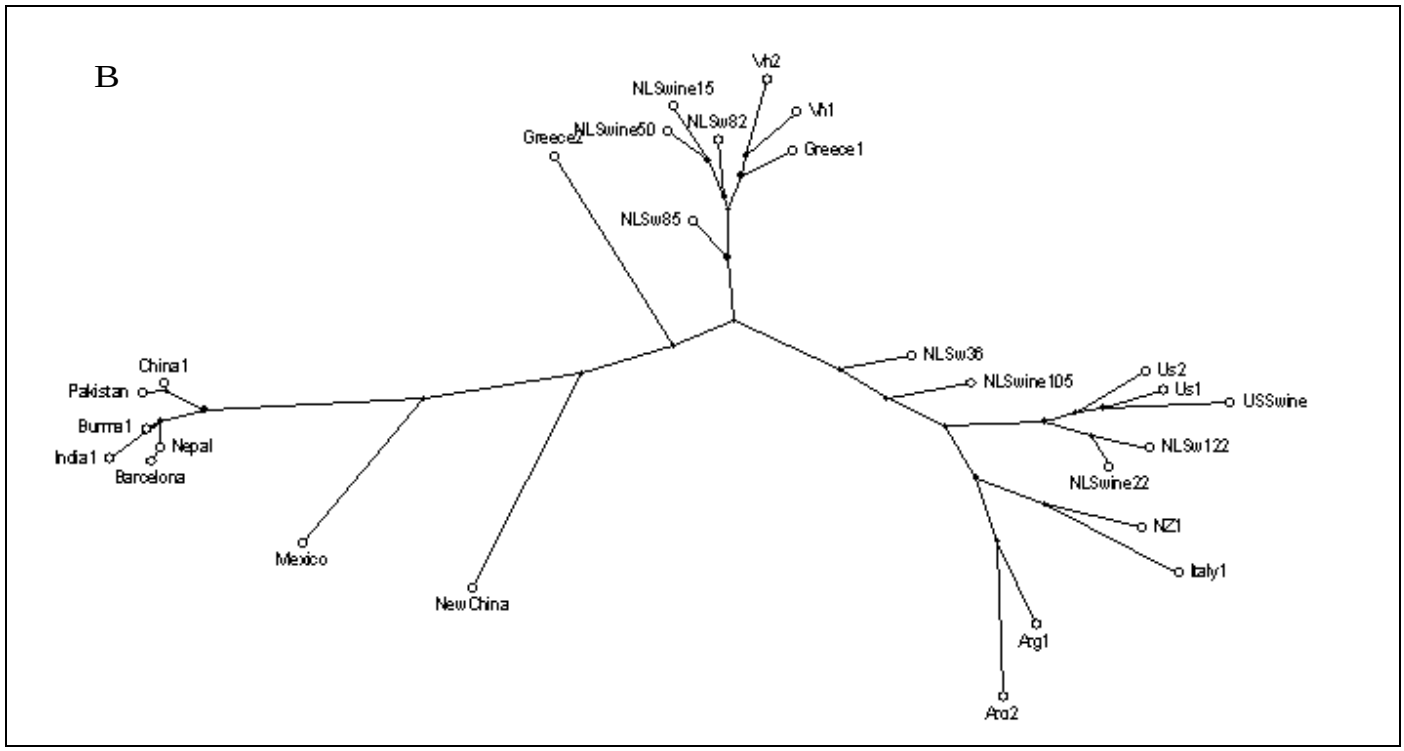


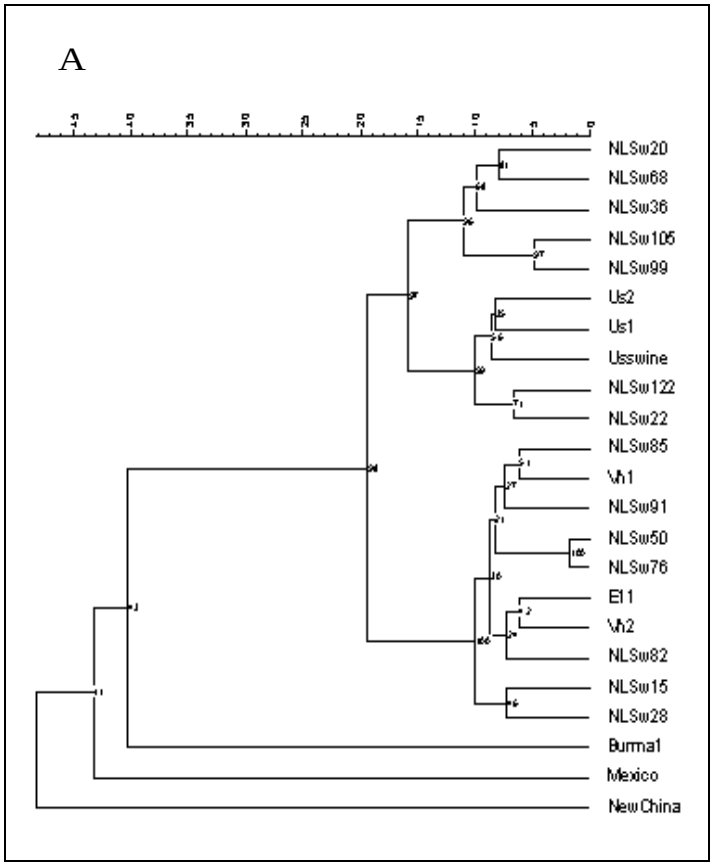

Other studies with different designs will be needed to find out whether HEV can cause clinical disease in swine. HEV may run a subclinical course in swine (7), a situation resembling the mostly asymptomatic hepatitis A and E infections in children (4). The outcome of natural HEV infection in adult and pregnant pigs is unknown and needs to be evaluated. In addition, it is unknown whether subclinical HEV infection may have adverse effects on growth rates in juvenile pigs.

On the basis of sequence comparisons, genetic distances, and phylogenetic analyses of the 242 bases of ORF1 and the 304 bases of ORF2, all swine HEVs in the Netherlands clustered with previously described European or American human or swine HEV isolates. There appears to be geographic clustering of swine and human sequences in Europe, America, and Asia. Only one Asian isolate (from New Zealand) clustered with a European isolate. The observation that American, European, and Asian human and swine isolates group together suggests relatively recent interspecies transmission in different parts of the world. To determine whether human HEV evolved from swine HEV or vice versa, the retrospective studies of archived fecal or serum samples from humans and swine may provide information about the evolutionary relationship between swine and human HEV.

An important issue raised from the discovery of swine HEV strains similar to human strains is the possibility of actual zoonotic transmission from swine to

Figure 2. Phylogenetic relationships among human and pig strains of *Hepatitis E virus* (HEV), based on a 242-bp sequence of ORF1 (nucleotides 125-366). Rooted tree (A) and unrooted tree (B). In the rooted tree, all the Dutch swine sequences are depicted with the foreign isolates that cluster with those sequences, as well as prototype isolates from different clusters. The distances can be estimated by using the scale, and the numbers are confidentiality rates. In the unrooted tree, eight Dutch swine sequences selected on the basis of diversity are depicted with isolates from different geographic origins. The numbers correspond with distances. NLSw15, 20, 22, 28, 36, 50, 68, 76, 82, 85, 91, 99, 105, and 122: 14 Dutch pig HEV. US1, US2 and USswine: United States human and pig HEV strains, respectively (19). Greece 1 and Greece 2: Greek human HEV strains (13). Arg1 and Arg 2: Argentinian human isolates (20). VH1 and VH2: Spanish human isolates; and E11: Spanish slaughterhouse sewage isolate (21). Burma1: Burmese human isolate (22). Italy1: human isolate (13). Barcelona: sewage isolate (23). Pakistan: human isolate (24). China1: human isolate (25). India1: human isolate (GenBank acc. no. X99441). NZ1: New Zealand human isolate (GenBank acc. no AF215661). Nepal: human isolate (26). Egypt: human isolate (22). Morocco: human isolate (7). NewChina (T1):GenBank acc. No AJ272108).





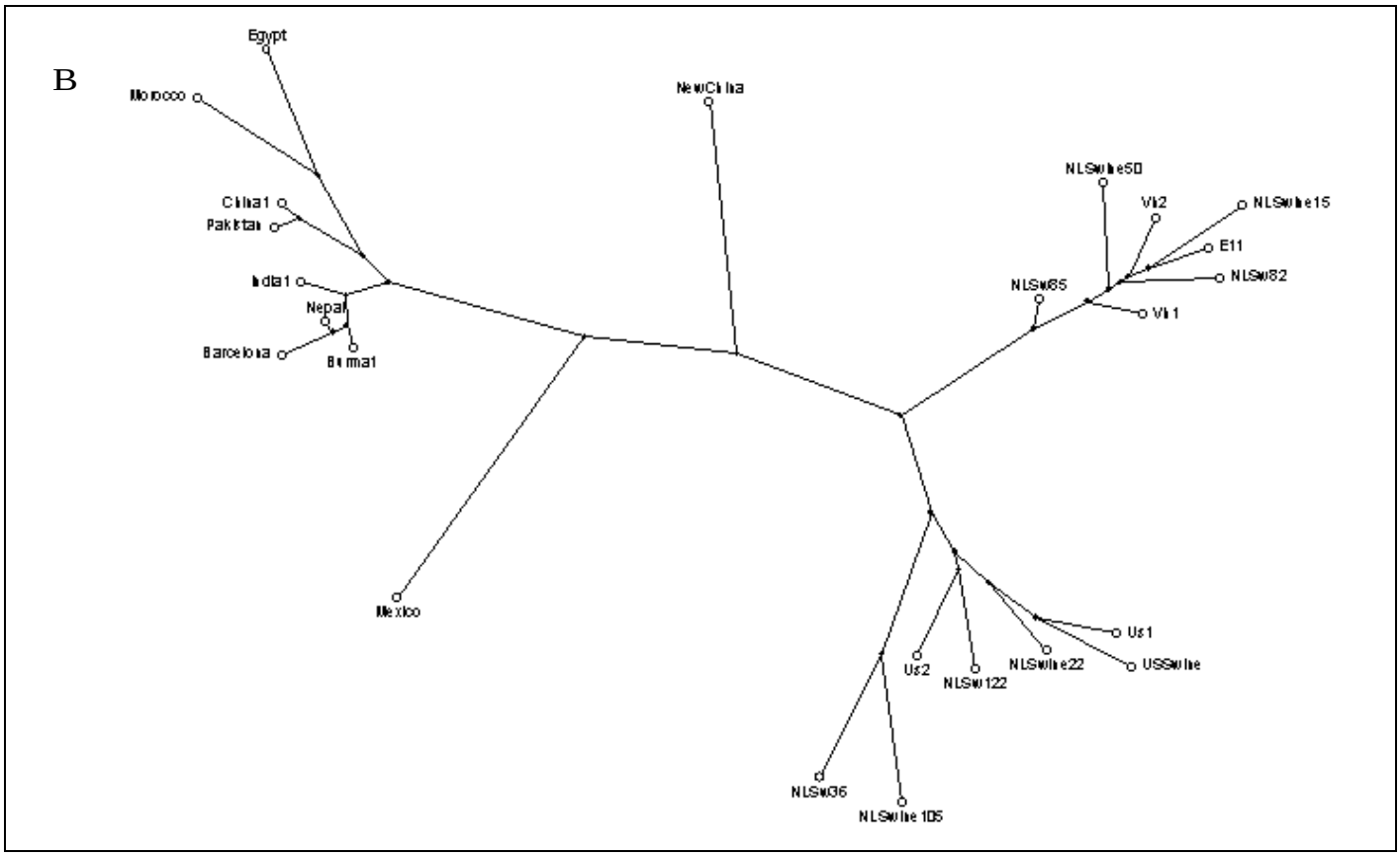

humans. Based on the sequence similarities observed among the Dutch swine HEV strains and the European and North American human strains, one cannot yet determine whether these swine strains are species-specific or circulating in the human population as well. Swine may be a reservoir for human infection. A reported higher anti-HEV seroprevalence among pig farmers working in close contact with pigs versus persons whose work does not involve contact with livestock (28) suggests that swine HEV may infect humans. If zoonotic HEV infections occur, whether HEV from swine can cause clinical disease in humans warrants study. Clinical HEV infection in the Netherlands in persons without a history of travel has not yet been observed; however, nontravelers with hepatitis are seldom tested for HEV.

HEV has been detected in sewage in Spain (23). The discovery of HEV in swine in the Netherlands suggests that humans may become infected by contact with sewage of animal origin or even through contact with surface waters.

In addition to the public health concern about zoonosis, there is also the concern for xenozoonosis, the inadvertent transmission of pathogens from animal organs to human recipients. Nonpathogenic pig HEV strains may become pathogenic for humans after xenotransplantation, as a result of species jumping, recombination, or adaptation in immunocompromised xenotransplantation recipients (29).

In conclusion, the discovery of swine HEV strains in

Figure 3. Phylogenetic relationships among human and pig *Hepatitis E virus* strains, based on 304 nucleotide sequences of HEV ORF2 (nu-cleotides 5994-6297). Rooted tree (A) and unrooted tree (B). For further explanation of the figure and definition of isolate acronyms, see Figure 2 legend.



the Netherlands related to human HEV isolates from Europe and America indicates an important new direction for HEV research. From the public health point of view, methods should be developed to detect interspecies transmission at an early stage. Swine HEV infection may provide an animal model for HEV studies, and swine HEV might also prove useful for development of a vaccine against human hepatitis E.

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# A Multistate Outbreak of *Escherichia coli* O157:H7 Infections Linked to Alfalfa Sprouts Grown from Contaminated Seeds

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A multistate outbreak of *Escherichia coli* O157:H7 infections occurred in the United States in June and July 1997. Two concurrent outbreaks were investigated through independent case-control studies in Michigan and Virginia and by subtyping isolates with pulsed-field gel electrophoresis (PFGE). Isolates from 85 persons were indistinguishable by PFGE. Alfalfa sprouts were the only exposure associated with *E. coli* O157:H7 infection in both Michigan and Virginia. Seeds used for sprouting were traced back to one common lot harvested in Idaho. New subtyping tools such as PFGE used in this investigation are essential to link isolated infections to a single outbreak.

*Escherichia coli* O157:H7 was first recognized as a human pathogen in 1982 and has since emerged as a major cause of bloody and nonbloody diarrhea, causing thousands of infections with substantial illness and death each year in the United States (1). In addition, *E. coli* O157:H7 infection is the most common cause of hemolytic uremic syndrome, the leading cause of kidney failure among children in the United States.

Most foodborne outbreaks associated with *E. coli* O157:H7 have been traced to foods derived from cattle, especially ground beef and milk (1). In June and July 1997, the state health departments of Michigan and Virginia concurrently received an increased number of reports of *E. coli* O157:H7 infections compared with the numbers in similar periods the previous year. We describe the epidemiologic, environmental, and laboratory investigations that led to the identification of alfalfa sprouts grown from contaminated seeds as a new vehicle for *E. coli* O157:H7 infection in humans.

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## Methods

### Epidemiologic Investigation

Independent studies were done in Michigan and Virginia to investigate exposures associated with *E. coli* O157:H7 infection.

#### Michigan

Cases were identified through passive surveillance of isolates sent from clinical laboratories to the Michigan Department of Community Health (MDCH). During July 21-27, 1997, we conducted a case-control study in Michigan, using a questionnaire designed to test hypotheses developed during in-depth interviews with seven ill persons. Because alfalfa sprouts and salads were the most commonly mentioned items in the hypothesis-generating interviews, the

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questionnaire focused on these food items. Other food items known to be vehicles for *E. coli* O157:H7 infections were also included.

A case was defined as diarrhea, abdominal cramps, or both, in a resident of Michigan with onset of symptoms from June 15 to July 31, 1997, and a stool culture yielding *E. coli* O157:H7 with the outbreak strain pulsed-field gel electrophoresis (PFGE) pattern. Ill persons were interviewed by telephone about their illness and exposures during the 7 days before onset of symptoms. Two age group-, sex-, and neighborhood- (based on the same telephone prefix) matched controls were selected for each patient. Matching by age was based on six groups ($\leq$2, 2 to <5, 5 to <12, 12 to <18, 18 to <60, and $\geq$60 years). Children <12 years of age were not matched by sex. Controls were identified by systematically adding to or subtracting from the case-patient's telephone number until a match was obtained. Controls were asked about food exposures during the 7 days before the day of interview. In addition, questions were asked about the consumption of selected food items such as alfalfa sprouts and salad during the time period including the 7 days before the onset of illness in the matched patient. The water quality records of a nearby lake where ill persons had swum were reviewed.

### Virginia

Cases were reported to the Virginia Department of Health by the local health districts, the state public health laboratory (Division of Consolidated Laboratory Services), and hospital and private laboratories. We initiated a case-control study on July 15, 1997, using a questionnaire regarding 31 specific food items and environmental exposures. The study design and questionnaire were not discussed with the Michigan investigators. Case-patients and controls were interviewed via telephone by trained interviewers who used a data collection instrument based on a Centers for Disease Control and Prevention (CDC) questionnaire used for a nationwide *E. coli* O157:H7 case-control study, with additional questions on items mentioned in interviews with ill persons. Controls were matched with case-patients by age, sex, and geographic location, with 1 to 3 controls per case. Case-patients <18 years old were matched within 3 years, those 18 to 34 years old within 5 years, and those >34 years old within 10 years. To locate geographically matched controls, investigators dialed the case-patients' area code and the first five digits of his or her telephone number and then completed the call by using a list of randomly generated numbers for the last two digits.

A confirmed case was defined as diarrheal illness (three or more loose stools in a 24-hour period) occurring in a Virginia resident (or nonresident if the person was seen in a Virginia health-care facility for treatment) with onset of symptoms from June 1 to September 5, 1997, and a stool culture that yielded *E. coli* O157:H7 with the outbreak strain PFGE pattern. Potential controls were excluded if they reported having had diarrhea in the 2 months before the interview or if they were not living in their current residence during the week before the matching patient's onset of illness. Controls were asked about the same time period as the matched patient. If case-patients or controls could not remember eating an item during that week, they were asked whether that item would have been eaten in a typical week in the month of the patient's illness onset.

### Laboratory Methods

PFGE subtyping of *E. coli* O157:H7 isolates was performed by the MDCH laboratory and the Virginia State laboratory by using the restriction enzyme *Xba*I (Boehringer Mannheim, Indianapolis, IN) as described (2). A subset of isolates (12 from Michigan and 24 from Virginia) were sent to CDC for simultaneous PFGE subtyping, phage typing, and antimicrobial susceptibility testing. PFGE subtyping of selected isolates was also performed by using the restriction enzymes *Bln*I (Boehringer) and *Spe*I (Boehringer).

In addition, we asked all state and territorial epidemiologists and public health laboratory directors to send to CDC *E. coli* O157:H7 isolates from any patients who had eaten alfalfa sprouts in the week before illness.

Isolates with PFGE patterns indistinguishable from the predominant pattern of isolates in Michigan were considered the outbreak strain. The outbreak PFGE pattern was compared with PFGE patterns in the CDC database by Molecular Analyst Fingerprinting Plus Software (Bio-Rad Laboratories, Hercules, CA). Phage typing was done by the extended phage typing scheme of Khakhria et al. (3). Isolates were tested by the disk-diffusion technique for susceptibility to the following antimicrobial agents: amoxicillin/clavulanic acid, ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole (4).

Implicated alfalfa seed and sprouts grown from that seed were cultured for *E. coli* O157:H7 in two enrichment broths (mTSB and TSBcv) (5,6). The broths were streaked to Sorbitol MacConkey Agar (CT-SMAC) containing 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite) and further examined by immunomagnetic separation (anti-*E. coli* O157 Dynabeads; Dynal, Inc., Lake Success, NY.) (7). The concentrated samples were then plated to selective media (CT-SMAC). Sweeps of growth on all plates were tested by polymerase chain reaction for stx1, stx2, and uidA gene sequences (8,9).

### Trace-Back Methods

State health departments, in conjunction with the Department of Agriculture in Michigan, the local Food and Drug Administration in Virginia, and CDC investigators, conducted interviews to determine the source(s) of sprouts. Ill persons identified retail outlets (restaurants, markets) and dates of purchase. Retail outlets identified shippers and growers of alfalfa seed. The origin of implicated seed was determined by reviewing seed supplier invoices and delivery records. A successful trace-back from a patient who had eaten sprouts was defined as one in which the grower and seed supplier(s) could be identified. After the alfalfa sprouts were traced back to one specific lot of seeds, we investigated the seed processing company and the fields from which they were harvested.

### Statistical Methods

Maximum likelihood estimates of matched odds ratios (MOR) with exact 95% confidence intervals (CI) were used as

measures of association (SAS statistical software version 6.12, SAS Institute Inc., Cary, NC, USA).

## Results

### Epidemiologic Investigation

#### Michigan

From June 1 to July 31, 64 persons with *E. coli* O157:H7 infection from Michigan were reported to MDCH, a twofold increase from the 31 infections reported during the same 2 months in 1996. Thirty-eight patients had illness that met the case definition (Figure). Of these, 26 (68%) were female, and the median age was 31 years (Table). Ninety-five percent reported bloody diarrhea, 47% were hospitalized, and 11% had hemolytic uremic syndrome; none died. Sixty-six percent of patients reported that they received antibiotics; 74% reported the use of antimotility agents.

Twenty-seven case-control sets were interviewed; the remaining patients either could not be reached or were identified after the study ended. The only food item positively and significantly associated with illness was alfalfa sprouts. Fifteen (56%) of 27 ill persons reported eating alfalfa sprouts in the 7 days before onset of illness, but only 3 (6%) of 53 controls had eaten them in the 7 days before the interview (MOR 27; 95% CI 5-558). When controls were asked about alfalfa sprout consumption for the same 7-day interval as ill persons, a similar association was observed (4 [8%] of 53 controls; MOR 25; 95% CI 4-528). No other food item–or swimming—was positively associated with illness.

#### Virginia

In June 1997, 32 persons with *E. coli* O157:H7 infection were reported, compared with 11 cases in the same time period in 1996. Seventy-four persons with *E. coli* O157:H7 infection with dates of onset from June 1 to September 5, 1997, were reported. Forty-four (59%) had illnesses that met the case definition; the exact onset dates were reported for 42 (Figure). Demographic and clinical characteristics of patients were similar to those of Michigan patients (Table).

Twenty case-control sets could be contacted for the case-control study. As in Michigan, eating alfalfa sprouts was the only exposure positively and significantly associated with illness. Thirteen (68%) of 19 case-patients but only 6 (13%) of 45 controls reported eating alfalfa sprouts during the week before illness onset in the case or in a typical week in the month of the patient's illness onset (MOR 25; 95% CI 4-537). No other food item was significantly associated with illness.

#### Laboratory Results

In the subset of 34 *E. coli* O157:H7 isolates from case-patients in Michigan and Virginia submitted to CDC, all isolates had indistinguishable PFGE patterns with restriction enzyme *Xba*I and *Bln*I. Three isolates were also tested by

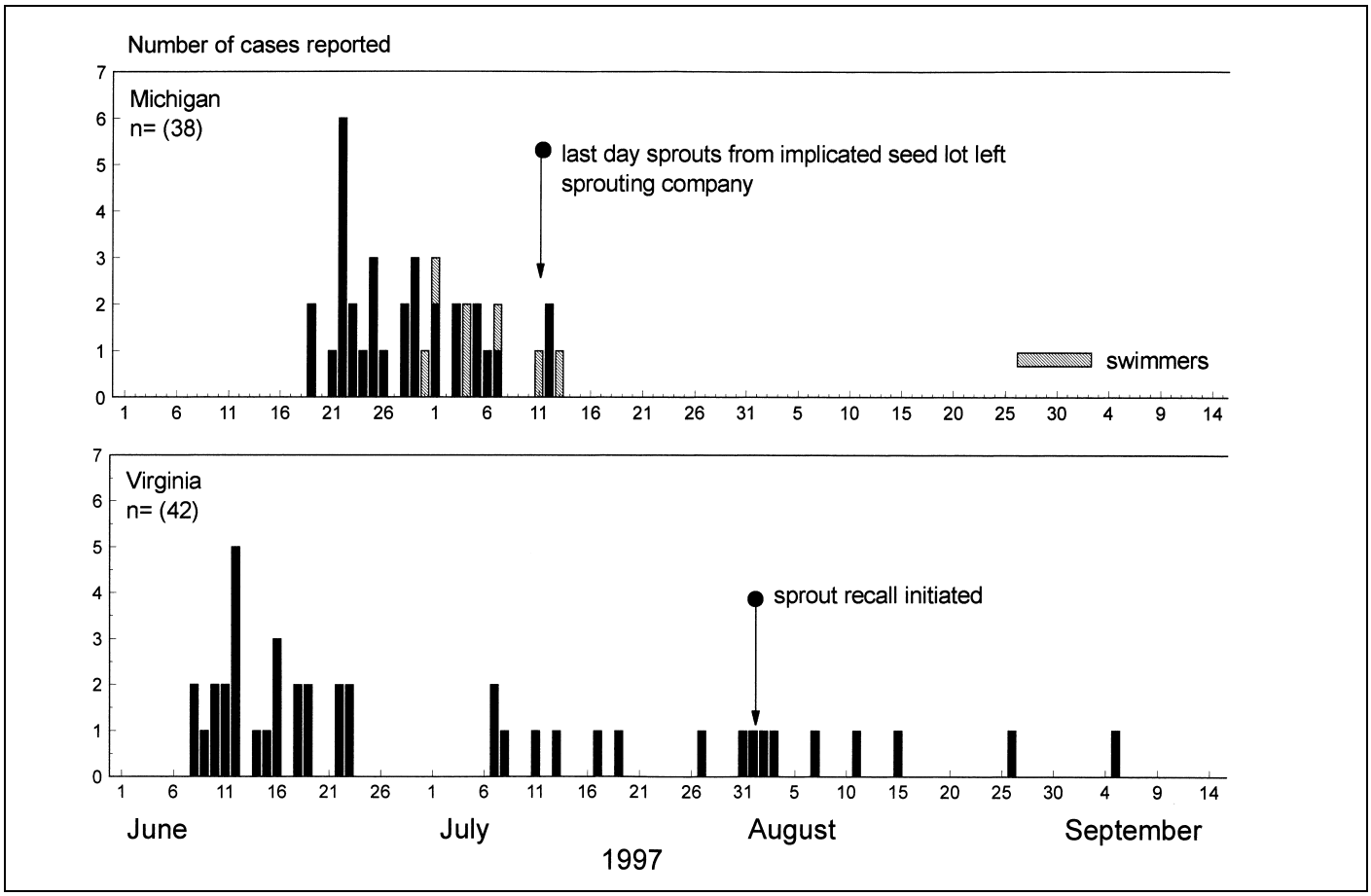


Figure. Date of onset of illness for persons with *Escherichia coli* O157:H7 infection and the outbreak pulsed-field gel electrophoresis pattern, Michigan and Virginia, June to September 1997.

Table. Demographic and clinical characteristics of persons with *Escherichia coli* O157:H7 infections and the outbreak pulsed-field gel electrophoresis patterns, Michigan and Virginia, 1997

| Characteristic | Michigan (n=38) | Virginia (n=44)[a] |
|---|---|---|
| Median age, years (range) | 31 (2-76) | 33 (1-71) |
| Female, no. (%) | 26 (68) | 26 (59) |
| Signs and symptoms, no. (%) | | |
| Diarrhea | 38 (100) | 44 (100) |
| Abdominal cramps | 37 (97) | 40 (100) |
| Bloody stool | 36 (95) | 38 (95) |
| Vomiting | 20 (53) | 26 (62) |
| Subjective fever | 15 (39) | 15 (43) |
| Hospitalized | 18 (47) | 18 (49) |
| HUS[b] | 4 (11) | 0 (0) |
| Headache | ND | 18 (47) |
| Muscle aches | ND | 15 (44) |
| Median days of diarrhea, no. (range) | 6 (3-41) | ND |

[a]Denominator ranged from 34 to 44 because of missing information.
[b]HUS = hemolytic uremic syndrome; ND = not determined.

*Spe*I and had indistinguishable patterns. All isolates were phage type 32. Thirty-three isolates were sensitive to all antimicrobial agents tested; one isolate from Virginia was resistant to ampicillin.

Sixty-seven isolates collected during the outbreak period from U.S. states other than Michigan and Virginia were tested at CDC by PFGE by using *Xba*I, and *Bln*I, and *Spe*I restriction enzymes. Six (9%) had PFGE patterns indistinguishable from the outbreak strain PFGE pattern by all three enzymes and were phage type 32, bringing the total number of cases to 85. These isolates came from Ohio and North Carolina, which are directly adjacent to the outbreak states.

We obtained a 50-pound bag of seeds used to grow the implicated alfalfa sprouts. A 500-g sample of seeds was cultured directly, and the same amount of seeds was sprouted; neither yielded *E. coli* O157:H7.

### Trace-Back

Trace-back to the sprouting facility was successful in 29 of 31 instances in which ill persons reported eating alfalfa sprouts. Of 16 successful trace-backs in Michigan, 15 led to one sprouting facility, facility A, in Michigan; one patient could have eaten sprouts from either facility A or facility B in Michigan. All 13 successful trace-backs in Virginia were traced to one sprouting facility in Virginia. During the outbreak period, the Virginia company used only one seed lot. That same seed lot was one of only two lots continuously sprouted by facility A in Michigan from mid-May to the first week of July. Facility B in Michigan sprouted a small number of seeds from this lot on only 2 days; the sprouts from these seeds represented only a fraction of each day's production. The implicated seed lot was not distributed to any other sprouting company in or outside the United States. That seed lot was 17,000 lbs, of which 6,000 lbs still existed and were immediately removed from distribution.

The implicated seed lot was a blend of five lots from four farms, harvested from 1984 to 1996. The seed processor and the farmers were all located in Idaho. Inspection of the alfalfa fields revealed three possible sources of contamination: cattle manure, water, and deer feces. Manure is not normally applied in alfalfa fields in Idaho; however, cattle feedlots are common in the area. The alfalfa fields of one of the farmers were adjacent to a feedlot. Manure may have leaked or been illegally dumped next to feedlots. In addition, run-off water from neighboring fields, which is collected in furrows and sometimes used to irrigate alfalfa fields, could carry manure to the fields. Three of the four farmers reported at least occasionally seeing deer in their fields. In fact, one had fields next to a wildlife refuge and reported that deer were in the fields every day. Contaminated alfalfa plants, cattle manure, or deer feces could be picked up by the thresher during harvesting and contaminate the seeds. No evidence was found for bacterial contamination at the seed processor.

### Other Sources of Illness

We interviewed 11 patients in Michigan who met the case definition but were not included in the case-control study because they either could not be contacted during the study or had not been identified when the case control-study was conducted. The median age of these patients was 12 years, compared with 31 years for patients in the case-control study. Their onsets of illness were from June 30 to July 13. Five of these patients, all children, had definitely not eaten alfalfa sprouts but had swum in the same manmade lake during the July Fourth holiday weekend or the previous weekend.

We re-interviewed the eight patients who were enrolled in the case-control study and who reported swimming in the 7 days before illness. We identified two children (ages 4 and 5) who had not eaten alfalfa sprouts in the week before illness but who had been swimming at the same manmade lake during the same time period. None of the other patients had visited the lake. The number of visitors per day was not higher on these weekends than the average number for weekend days in June and July. Water samples from three locations in the beach area of the lake contained $\leq$ 10 *E. coli* bacteria/100 mL on June 25 and July 7.

Demographic characteristics of patients in Virginia (n=21) who were not included in the case-control study did not differ from those of patients enrolled in the study.

### Follow-Up of Patients Outside the Outbreak States

We contacted all six ill persons from North Carolina and Ohio who were infected with *E. coli* O157:H7 of the outbreak strain PFGE pattern. Two of them had traveled to an outbreak state during the outbreak period but could not remember eating alfalfa sprouts. Three remembered eating alfalfa sprouts in the week before illness but did not recall traveling to an adjacent outbreak state during that time.

## Discussion

This multistate outbreak of *E. coli* O157:H7 infections is the first outbreak linked to consumption of alfalfa sprouts. It is also the first outbreak in which subtyping by PFGE was used to determine the magnitude of the outbreak on a

national scale. Many lines of evidence indicate that the vehicle was alfalfa sprouts grown from contaminated seed. First, two independently designed and conducted case-control studies of concurrent outbreaks in Michigan and Virginia found that eating alfalfa sprouts was the only exposure positively associated with illness. Second, *E. coli* O157:H7 isolates from Michigan and Virginia patients epidemiologically linked to the outbreak had an indistinguishable PFGE pattern, the same phage type, and the same antibiogram, strongly suggesting a common source. The identical PFGE pattern had been identified only once before in one isolate in CDC's database of PFGE patterns. Third, trace-back investigations implicated independently operating sprouting facilities in Michigan and Virginia. The only common link between these sprouting facilities was the use of the same seed lot, grown and shipped from Idaho, indicating that the seed was contaminated before it was shipped to the sprouting facilities. Fourth, the outbreak ended after alfalfa sprouts from the implicated seed lot were no longer sold. Fifth, a national sample of other *E. coli* O157:H7 isolates collected during the outbreak period did not contain the PFGE outbreak pattern, except for a few isolates from ill persons in states adjacent to the outbreak states, who could have eaten the implicated sprouts. The lack of a nationwide distribution of cases is consistent with the fact that the seeds and the sprouts grown from them were distributed in only two states.

This outbreak also highlights the use of PFGE subtyping as a tool for differentiating between an increase of sporadic unlinked infections and a cluster of infections from a common source. After an increased number of reports of *E. coli* O157:H7 infections was noticed, rapid PFGE subtyping of the initial isolates within 2 days was the starting point of this investigation.

The removal of 6,000 pounds of remaining seeds from the marketplace likely prevented more illnesses, although cultures of implicated seeds and sprouts grown from them did not yield *E. coli* O157:H7. Contamination was probably not uniform in the lot of seed from which the implicated sprouts were grown, and since only a single bag, representing <0.003% of that seed lot, was cultured, recovery of *E. coli* O157:H7 may have been unlikely. This outbreak investigation illustrates the recommendation that public health officials should not require confirmation of microbial contamination of a product before taking action when sufficient epidemiologic evidence is available.

In recent years, produce items such as lettuce, apple cider, and unpasteurized apple juice have been implicated in outbreaks of *E. coli* O157:H7 infections (10). Detection of non-meat-related outbreaks is most likely explained by heightened awareness of *E. coli* O157:H7 infection and improved detection methods such as PFGE subtyping, which enable recognition of smaller clusters and widely dispersed outbreaks. The expanded range of food vehicles also highlights the need for changes in educational messages and for improving awareness among physicians and the general public that *E. coli* O157:H7 infections can be acquired from many sources other than ground beef.

In recent years, consumption of raw alfalfa sprouts has also been associated with outbreaks due to various serotypes of *Salmonella* (11,12). Salmonellae can survive for months under the dry conditions used for alfalfa seed storage (13), and *E. coli* O157:H7 likely follows a similar survival pattern. Furthermore, *E. coli* O157:H7 proliferates $10^3$- to $10^5$-fold during sprout germination. Viable *E. coli* O157:H7 can exist not only on the outer surface but also inside the sprout vessels (14). If this finding is confirmed by other researchers, it is even more important to identify sources of contamination and institute specific prevention measures.

The U.S. sprouting industry produces several hundred thousand tons of sprouts of different varieties each year. No methods to reduce or eliminate contamination of seeds in the field, to effectively decontaminate alfalfa seeds before sprouting, or to clean the sprouts themselves are in place. The sprout industry is working with the National Center for Food Safety and Technology to study sprout safety. The most promising method is chemical treatment with calcium hypochlorite, a method already in use in California on an emergency basis, as approved by the state's environmental protection agency (15). Irradiation, in which a measured dose of ionizing radiation is applied, appears to work well in decontaminating sprout seeds; U.S. Food and Drug Administration approval is pending (15). Until the safety of alfalfa sprouts can be assured, we recommend that persons at increased risk for *E. coli* O157:H7 infections, such as children <5 years of age, the elderly, and patients with compromised immune systems, should not eat alfalfa sprouts (16,17).

This outbreak shows how foodborne outbreaks can extend in a community. The identification of an identical PFGE pattern in a second cluster of patients at the end of the outbreak suggests that a lake was contaminated by feces from a patient with illness from sprouts. Such contamination is possible because *E. coli* O157:H7 can survive for weeks in lake water (18) and has a very low infectious dose. Children could have acquired illness by swallowing a small amount of water while swimming (19,20). The finding of a probable waterborne outbreak also underscores the fact that new subtyping methods such as PFGE are tools to improve investigations but cannot substitute for a thorough epidemiologic work-up. Epidemiologic investigation combined with PFGE made the link to the source of the outbreak and to a specific lot of alfalfa seeds. Additional epidemiologic inquiry established lake water as the most likely mode of transmission for children who had not eaten sprouts.

Because food is increasingly centrally produced and widely distributed (21), the public health system will likely face more widely dispersed outbreaks such as this one. To meet the challenge, subtyping tools such as those used in this investigation, as well as timely and centralized disease reporting and outbreak investigation by trained public health personnel, the application of appropriate microbiologic tests in suspected cases, and increased awareness of foodborne illness will be essential.

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# Effect of Prevention Measures on Incidence of Human Listeriosis, France, 1987–1997

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To assess the impact of preventive measures by the food industry, we analyzed food monitoring data as well as trends in the incidence of listeriosis estimated through three independent sources: the National Reference Center of Listeriosis; a laboratory-based active surveillance network; and two consecutive nationwide surveys of public hospital laboratories. From 1987 to 1997, the incidence of listeriosis decreased by an estimated 68%. A substantial reduction in the proportion of *Listeria monocytogenes*-contaminated products was observed at the retail level. The temporal relationship between prevention measures by the food industry, reduction in *L. monocytogenes*-contaminated foodstuffs, and reduction in listeriosis incidence suggests a causal relationship and indicates that a substantial part of the reduction in illness is related to prevention efforts.

Although rare, invasive listeriosis is an infection of public health concern because of its severity, with a case-fatality rate evaluated at 20%-30%, the possible sequelae, and its potential to cause epidemics. In 1981, the investigation of an outbreak of listeriosis in Canada demonstrated for the first time that human listeriosis could be caused by transmission of *Listeria monocytogenes* through contaminated foods, in this case coleslaw (1). However, it was not until 1985, prompted by the outbreak of listeriosis in California linked to inadequately pasteurized soft cheese, that *L. monocytogenes* became a major concern of the food industry. In the United States, as a first step, the Food and Drug Administration began monitoring dairy products for *L. monocytogenes*. This monitoring was later expanded to include ready-to-eat foods such as cold meat and poultry products, seafood, and salads; strict zero-tolerance guidelines for *L. monocytogenes* in ready-to-eat foods were enforced (2).

In France, the first control measures for *L. monocytogenes*, including microbiologic monitoring of unprocessed and finished products, were implemented in 1986 in plants producing cheese for exportation to the United States (3). When a soft cheese was shown to be the cause of a large outbreak that occurred in Switzerland from 1983 to 1987, French authorities enforced prevention efforts to eliminate sources of contamination by *L. monocytogenes* in all cheese production plants beginning in 1988 (4). In 1992, *Listeria* control measures were extended to include plants producing ready-to-eat meat and meat products (5). In 1992, the investigation of a large outbreak (279 cases) identified pork tongue in jelly as the main vehicle of transmission and showed that cross-contamination of other ready-to-eat meat products, especially those sold at the delicatessen counter, amplified transmission (6). As a result, hygiene measures at the retail level and particularly at delicatessen counters were reinforced (7,8). This 1992 outbreak prompted the Ministry of Health to issue recommendations through the public media to immunocompromised patients, the elderly, and pregnant women to avoid certain foods, encouraging them to consult a physician in case of symptoms suggestive of listeriosis.

Finally, in 1993, *L. monocytogenes* control measures were extended to include all foods potentially contaminated with *L. monocytogenes* (9). These control measures included microbiologic monitoring of food products and, if *L. monocytogenes* were isolated, investigation, increased sanitation, and plant clean-up. Foods were considered to be potentially contaminated with *L. monocytogenes* if they were either raw and did not undergo a listericidal process (such as cooking) before being eaten, or if they were pasteurized foods that could be contaminated during handling and that have characteristics favorable to the growth of *Listeria* (e.g., pH, water content, and salt concentration within certain ranges). For some products (food for infants and toddlers, canned food) a zero-tolerance policy was enforced. For dairy, meat, and fish products, the standard is to have no contamination, but <100 CFU *L. monocytogenes*/g at the end of the product's shelf life can be tolerated under certain circumstances.

In the same period, the dairy corporation and meat processing association produced video training programs to help managers improve food hygiene understanding by educating their employees. Several guidelines were published, in 1991 and 1992 for the dairy industry and in 1994 for the meat product industry. Guidelines to introduce Hazard Analysis and Monitoring of Critical Control Points (HACCP) in manufacturing plants were promulgated in 1992 by the meat and

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dairy industry, and software was developed to help managers implement this method in their plants.

We analyze trends in the incidence of listeriosis to assess the impact of the measures described.

## Material and Methods

### Case Surveillance

Data on the incidence of human listeriosis in mainland France were obtained from three independent sources. The first source was the National Reference Center (NRC) of listeriosis, to which microbiology laboratories voluntarily send human isolates of *L. monocytogenes*. The NRC was the Microbiology Laboratory of the Medical Faculty in Nantes from 1982 to 1992; since 1993, the Institut Pasteur in Paris has performed this function. For patients identified by the NRC in 1997, information on pregnancy status and underlying medical conditions was obtained by contacting the attending physician (10).

The second source was two surveys carried out among public hospital laboratories to estimate listeriosis incidence in France in 1987 and 1988 (11,12). In France, general hospitals that provide primary care are distinguished from regional hospitals, usually university teaching hospitals, which provide primary as well as secondary care. With the rare exceptions of a few specialized university hospitals, all general and regional/university hospitals have neonatal or pediatric departments or both, as well as an obstetric department.

In the retrospective surveys, all laboratories of general and regional/university hospitals were asked to complete case reports for all patients from whom *L. monocytogenes* had been isolated in the year of the survey. Information was collected on the site of isolation of the strain and age and sex of the patient. In 1987, information on pregnancy status and underlying disease, as well as clinical outcome of listeriosis, was also recorded. For each year, the total number of cases in France was estimated by dividing the number of cases reported by the participating hospitals by the proportion of beds represented by these hospitals (number of beds in participating hospitals/total number of beds in all public hospitals in France).

The third source was a surveillance network of laboratories of general and regional/university hospitals called EPIBAC, which has been operational since 1987 (13). Participating laboratories complete monthly report forms indicating all cases of bacteremia or meningitis with an isolate of *L. monocytogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, or *Streptococcus* groups A and B. For each case, the type of isolate and the date of birth and sex of the patient are recorded. Until 1991, a case was defined as an isolate. Therefore, a patient with isolates from both blood and cerebrospinal fluid (CSF) was reported on two separate forms as a case of bacteremia and a case of meningitis. Since 1991, cases of listeriosis are reported on a single form, and for each case it is noted whether the isolate was recovered from blood or CSF. Consequently, for the period 1987-1997, we cannot analyze trends in incidence from the total number of cases, since the number of cases before 1991 would be overestimated. Therefore, we analyzed trends in incidence from 1987 to 1997 separately from the total number of blood isolates (whether or not associated with an isolate from CSF) and from the total number of CSF isolates (whether or not associated with an isolate from blood).

For each year, the total number of cases of listeriosis in France was estimated by dividing the number of cases reported by the participating hospitals by the proportion of hospital admissions in medicine departments (all nonsurgical departments) represented by these hospitals (number of admissions in medicine departments of participating hospitals/total number of admissions in medicine departments in public hospitals in France).

### Case Definition for Surveillance

In the NRC and in the two surveys of hospital laboratories, a case of listeriosis was defined as isolation of *L. monocytogenes* from a normally sterile site or, in the case of a newborn <7 days of age, from any site. The EPIBAC network, which used a more restricted case definition, reported only invasive cases with an isolate of *L. monocytogenes* from blood or CSF. Patients with blood or CSF isolates represent approximately 80% of all cases identified by laboratory surveys and the NRC. Pregnancy-associated cases were defined by the isolation of *L. monocytogenes* from a pregnant woman or her fetus or newborn infant within the first 30 days of life. A mother-infant pair (even in the case of twins) was counted as a single case. A predisposing condition was defined as one for which an increased risk of listeriosis had been demonstrated in a previous study (14; manuscript in preparation). These conditions included malignancies, chronic hemodialysis, hepatic or renal failure, diabetes, HIV/AIDS, corticotherapy, chemotherapy, and organ transplantation.

### Sensitivity of Data Sources

The number of laboratories sending isolates to the NRC increased over time. In 1986, comparison with the number of cases identified by a survey among hospital laboratories estimated this proportion at <33% of the isolates (15). For 1997, the sensitivity for case-detection by EPIBAC and NRC was evaluated by the capture-recapture method (16). A total of 225 cases with *L. monocytogenenes* isolated from blood or CSF were identified by one or both systems: 148 by EPIBAC, 190 by NRC, and 113 by both systems. The sensitivity for detecting cases of bacteremia or meningitis was estimated at 76% (95% confidence interval [CI] 72-81) for NRC and 59% (95% CI 56-63) for EPIBAC. The annual incidence of invasive listeriosis in 1997 was thus estimated for NRC by dividing the number of identified cases by 76%.

### Food Monitoring

In France, two ministries are responsible for monitoring foods for *L. monocytogenes*. The Ministry of Agriculture is responsible for monitoring at the production level. In addition to inspecting sanitary conditions in processing plants, its officers test foods bacteriologically at different phases of production and review results of microbiologic surveillance carried out by the producer. Until 1998, results of these tests were not available for analysis, since the data remained at the local level.

The Ministry of Economy (Consumers Affairs Directorate) conducts 2-year surveys to determine the proportion and level of contamination of foodstuffs considered to be at risk for *L. monocytogenes* contamination and to evaluate the impact of preventive efforts on the frequency of

contamination of these foodstuffs. The results of these surveys are available from 1993 on (17,18). The surveys focus on foods considered to be at risk for *L. monocytogenes* contamination because of characteristics favoring its growth; ready-to-eat foods with a long shelf life are of particular interest. These high-risk foods are divided into four categories (meat, dairy, seafood-fish products, and prepared salads), which are again divided into subcategories of food types that are considered comparable with respect to the likelihood of contamination. Dairy products, for example, are divided into subcategories according to whether the product is pasteurized; soft or hard; ripened or fresh; made of cow, goat, or sheep milk; or blue veined or white mold. In the 1993-94 and 1995-96 surveys, products from 27 subcategories of ready-to-eat foods were sampled at the retail level so that the distribution of the subcategories was similar in both surveys. For these ready-to-eat products, we compared the proportion of samples within the four categories of foods that was contaminated in the periods 1993-1994 and 1995-1996.

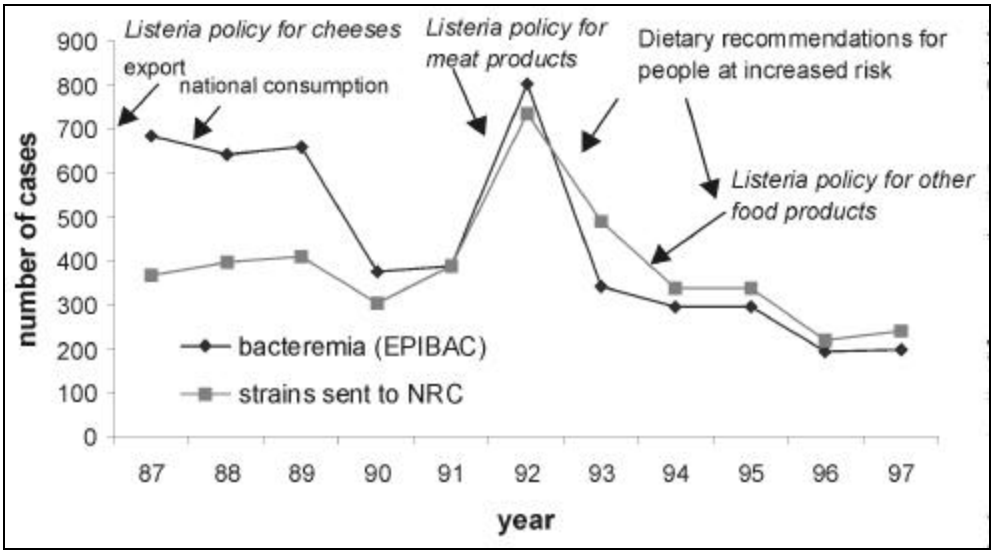


Figure. Annual incidence of *Listeria monocytogenes* bacteremia estimated by the EPIBAC laboratory network, number of isolates received by the National Reference Center, and year of implementation of preventive measures, France, 1987-1997.

### Statistical Analysis

Incidence data were calculated by using the census data estimates from Institut National de la Statistique et des Etudes Economiques (19). For 1987 and 1997, the distribution by category of the identified patients with information on pregnancy status and medical conditions was applied to the total estimated annual incidence to obtain category-specific incidence rates. Changes in incidence by category were evaluated by comparing the category-specific incidence rate in 1987, as estimated by the 1987 hospital laboratory survey, with the category-specific estimated incidence rate in 1997, based on the number of isolates received by NRC.

Since the 1987 survey used a different approach from the 1997 study, we also analyzed the proportional reduction in number of cases by category, from the subset of hospitals that reported cases in both 1997 and 1987. Proportions were compared by chi-square test with Yates correction or Fisher exact test, as appropriate.

## Results

### Incidence of Listeriosis (Figure)

#### EPIBAC

From 1987 to 1993 and from 1994 to 1997, participating hospitals represented 35% and 60%, respectively, of public hospital admissions in medicine departments in France. Participation of regional/university hospitals was slightly but consistently higher than that of the general hospitals: in EPIBAC hospitals, 42% of hospital admissions were in university/regional hospitals; the figure for the whole country is 37.5%. The number of admissions in pediatric departments divided by the number of admissions in all medicine departments (all nonsurgical) was consistently the same (14%) in EPIBAC hospitals and in all hospitals in France.

Using data from the EPIBAC network, we found the annual estimated incidence of bacteremia and meningitis due to *L. monocytogenes* declined substantially, from 12.3 cases for bacteremia and 3.4 cases of meningitis per million population in 1987 to 3.5 cases of bacteremia and 0.9 cases for meningitis per million population in 1997, a reduction of 72% and 73%, respectively. The incidence decreased slightly from 1987 to 1989, showed a clear decrease in 1990, and continued to diminish until 1997, with the exception of 1992, when a large outbreak involving 279 cases occurred in France.

The annual number of cases identified by NRC decreased by 33% from 366 (6.3 cases/million) in 1987 to 242 (4.1 cases per million) in 1997. The trend is similar to that in EPIBAC: a decrease was observed in 1990, followed by a peak in 1992, and then a decrease after 1993. The number of cases identified by the NRC in 1994-1995 was similar to the number identified in 1988-1989, whereas the incidence estimated by EPIBAC shows a 54% reduction over this period.

### Hospital Laboratory Surveys

Overall, the hospitals participating in the hospital surveys represented 71% (1987) and 74% (1988) of all public hospitals beds in France. The participation of regional/university hospitals was higher than that of general hospitals: in 1987 and 1988, the participating regional/university hospitals represented 77% and 83%, respectively, of the regional/university hospital beds in France; the participating general hospitals represented 67% and 70%, respectively, of the general hospital beds in France. The proportion of pregnancy-associated cases was similar in regional/university hospitals (53%) and general hospitals (49%).

Through these surveys, the incidence of listeriosis was estimated in 1987 at 16.7 and in 1988 at 14.9 cases/million population. The surveys show a decrease in incidence of 11% from 1987 to 1988, slightly higher than the 6% decrease observed by EPIBAC over the same period.

Table 1. Incidence and proportion of listeriosis cases by category of patient in 1987 and 1997, France

| Patient categories | Cases estimated per 1,000,000 population | | Percent change | % of cases | |
|---|---|---|---|---|---|
| | 1987[a] | 1997[b] | % | 1987[a] | 1997[b] |
| Pregnancy-associated | 8.5 | 1.3 | -84 | 51 | 24 |
| <65 years of age with no predisposing condition | 1.7 | 0.3 | -82 | 10 | 6 |
| ≥65 years of age without predisposing condition | 1.3 | 0.5 | -62 | 8 | 9 |
| Presence of predisposing condition | 5.3 | 3.3 | -37 | 31 | 61 |
| Total | 16.7 | 5.4 | -68 | 100 | 100 |

[a] Hospital laboratory survey.
[b] National Reference Center

Table 2. Number of listeriosis cases by category of patient in 1987 and 1997, France: comparison of results from the total data set of two surveys and from a subset of hospitals reporting cases in both surveys

| Patient category | All hospitals | | | Subset of hospitals reporting cases in 1987 and 1997 | | |
|---|---|---|---|---|---|---|
| | Cases identified | | | Cases identified | | |
| | 1987[a] | 1997 [b] | Percent change[c] | 1987[a] | 1997[b] | Percent change |
| Pregnancy-associated | 336 | 58 | -83 | 166 | 25 | -85 |
| No predisposing condition; <65 years of age | 66 | 13 | -80 | 36 | 6 | -84 |
| Predisposing condition; ≥65 years of age | 51 | 22 | -57 | 19 | 8 | -58 |
| Predisposing condition | 208 | 148 | -29 | 112 | 85 | -24 |
| Total | 661 | 241 | -64 | 333 | 124 | -63 |

[a] Hospital laboratory survey.
[b] National Reference Center.
[c] The percent change in number of cases has been calculated instead of the percent change in incidence, to facilitate comparison w ith the figures from the subset of hospitals. As a result, the figures on percent change are slightly lower than those in Table 1, which take into account the increase in the French population during the 10-year period.

### Incidence by Category of Patient

The decrease in incidence was most prominent among pregnant women (84%) and persons <65 years of age without predisposing conditions (82%) (Table 1). The decrease was less marked among elderly subjects (≥65 years of age) without predisposing condition (62%) and much lower among persons with a predisposing condition (37%). Analysis of the subset of hospitals that participated in both the 1987 and the 1997 study yielded very similar results as the analysis of the total data set (Table 2). As a result, the relative importance of different categories of patients changed drastically. In 1987, 51% of the cases were pregnancy associated, but in 1997 this category represented 24% (Table 1). Patients with a predisposing condition represented only 31% of all cases in 1987 but accounted for 61% of all cases in 1997.

### Food Monitoring

In 1993-94 and in 1995-96, a total of 5,809 and 6,147 ready-to-eat products were sampled, respectively. Overall, meat products (10.8%) and seafood-fish products (10.4%) were contaminated more frequently than dairy products (4.7%) and prepared salads (4.5%). However, dairy products were more frequently contaminated at higher doses than other products: 1.8% of dairy products were found to be contaminated with ≥100 CFU/g, compared with 0.3%, 0.5%, and 1.1% for salads, seafood/fish products, and meat products, respectively (Table 3).

During 1993-94 and 1995-96, we observed a decrease in the proportion of contaminated products that was greater for products contaminated with ≥100 CFU/g (38%) than for products contaminated with <100 CFU/g (10%) (Table 4). The decrease in the proportion of products with contamination ≥100 CFU/g was 56% for dairy products and 41% for meat products. Foods with levels of contamination <100 CFU/g decreased by 33% for prepared salads and 23% for meat products, but no substantial change was observed for dairy products and seafood-fish products.

## Discussion

The results indicate that the incidence of invasive disease by *L. monocytogenes* decreased substantially from 1987 through 1997. The reduction in incidence was 72% when estimated through the EPIBAC network and 68% when estimated by comparing incidence rates from the 1987 hospital laboratory survey and the NRC in 1997. Since the 1987 survey used a different approach from the 1997 study, the difference in sensitivity of the two approaches may have resulted in or contributed to the reduction observed. However, analysis of the subset of hospitals that participated in both the 1987 and 1997 studies provided comparable data and yielded results very similar to those of the analysis of the total data set. The number of cases identified through the public hospital laboratory surveys also decreased from 1987 to 1988, consistent with the trend observed by the EPIBAC network.

Table 3. Proportion of ready-to-eat foods contaminated with *Listeria monocytogenes* (Lm), by food category, France, 1993-1996

| | Ready-to-eat meat products | Dairy products | Prepared salads | Seafood-fish products |
|---|---|---|---|---|
| Number of samples | 3,283 | 3,541 | 3,166 | 1,966 |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[a] | 9.7 | 2.9 | 4.2 | 9.7 |
| ≥100 CFU/g | 1.1 | 1.8 | 0.3 | 0.5 |
| Total | 10.8 | 4.7 | 4.5 | 10.4 |

[a]Levels of contamination <100 CFU/g but greater than zero.

Table 4. Proportion of ready-to-eat foods contaminated with *Listeria monocytogenes* (Lm) by food category, level of contamination, and period, France, 1993-1996

| | 1993-94 | 1995-96 | Percent change (%) | p[a] |
|---|---|---|---|---|
| **Ready-to-eat meat products** | | | | |
| Number of samples | 1,533 | 1,750 | | |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[b] | 11 | 8.5 | -23 | 0.02 |
| ≥100 CFU/g | 1.6 | 0.7 | -56 | 0.03 |
| Total | 12.6 | 9.2 | -28 | 0.003 |
| **Dairy products** | | | | |
| Number of samples | 1,695 | 1,846 | | |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[b] | 3.2 | 2.7 | -16 | ns |
| ≥100 CFU/g | 2.2 | 1.3 | -41 | 0.03 |
| Total | 5.4 | 4 | -26 | 0.03 |
| **Prepared salads** | | | | |
| Number of samples | 1,740 | 1,426 | | |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[b] | 4.9 | 3.3 | -33 | 0.02 |
| ≥100 CFU/g | 0.3 | 0.3 | 0 | ns |
| Total | 5.2 | 3.6 | -31 | 0.02 |
| **Seafood–fish products** | | | | |
| Number of samples | 841 | 1,125 | | |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[b] | 9.3 | 11.4 | +22 | ns |
| ≥100 CFU/g | 0.7 | 0.5 | -29 | ns |
| **Total** | 10 | 10.3 | +18 | ns |
| Total no. of samples | 5,809 | 6,147 | | |
| % of food contaminated by Lm | | | | |
| <100 CFU/g[b] | 6.6 | 5.9 | -10 | ns |
| ≥100 CFU/g | 1.3 | 0.8 | -38 | 0.007 |
| Total | 7.9 | 6.7 | -16 | 0.008 |

[a]Chi-square test with Yates correction or Fisher exact test.
[b]Levels of contamination <100 CFU/g but greater than zero.

Data on the incidence by category of patient clearly indicate that the largest reduction in incidence occurred in pregnant women and previously healthy adults <65 years of age. A similar observation was made in the United States from 1989 to 1993, where an overall reduction in incidence of 44% was observed (51% in pregnancy-associated cases and 37% in patients >50 years of age), and in England and Wales, where the proportion of pregnancy-associated cases declined from 31%-48% between 1983 and 1989 to 8%-26% between 1990 and 1996 (2,20).

During the period that the decrease in incidence was observed, prevention measures have been progressively introduced by the food industry. Food monitoring data strongly suggest that these measures have successfully reduced the distribution of *L. monocytogenes*-contaminated ready-to-eat foodstuffs. The temporal relationship between the preventive measures, the reduction in *L. monocytogenes*-contaminated foodstuffs, and the decrease in incidence supports a causal relationship. A similar temporal association of reduction in incidence with implementation of prevention measures was observed in the U.S. study (2). The incidence of listeriosis per million population, much higher in 1986 in France (14.7) than in the United States (7.3), was similar in 1997 (France 5.4; USA 4.8) (21-23). Thus, the reduction in rate from 1986 to 1997 was much greater in France.

Dietary recommendations for immunocompromised patients and pregnant women to avoid certain foods may have contributed to the decline in incidence. However, several findings suggest that this contribution is unlikely to have been substantial. First, although dietary recommendations were targeted only at immunocompromised persons, the elderly, and pregnant women—never at previously healthy adults <65 years old—an 83% reduction in incidence was observed in this category. In addition, incidence began to decline in the period 1987 to 1992, when prevention measures at the production level were the only steps taken to prevent listeriosis in France.

Food monitoring data indicate that the reduction in *L. monocytogenes*-contaminated products was greatest for more heavily contaminated products, with an overall decrease of 38% for products contaminated at $\geq$100 CFU/g, compared with 10% for products contaminated at <100 CFU/g. The findings that the decrease in contaminated products was most pronounced for the more heavily contaminated products and that the reduction in incidence was most important among previously healthy adults and pregnancy-associated cases support the hypothesis that these two groups need to be exposed to a higher infectious dose for invasive illness to develop, as demonstrated by dose-response curves (24). This hypothesis is consistent with the finding that, in several outbreaks in France linked to a highly contaminated food, previously healthy adults and pregnancy-associated cases represent a far larger proportion of cases than among sporadic cases of listeriosis (25,26).

In summary, in France, the incidence of invasive disease due to *L. monocytogenes* decreased by an estimated 68% from 1987 to 1997. The decrease started during 1987-1992, when measures by the food industry were the only steps taken in France to prevent listeriosis. The decrease in incidence was particularly important in previously healthy adults, not included in dietary recommendations. These findings suggest that a substantial part of the decrease in illness due to *L. monocytogenes* is related to control measures implemented at the food production level.

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# The Changing Epidemiology of Leptospirosis in Israel

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We reviewed all serologically confirmed cases of leptospirosis from 1985 to 1999 in Israel, where the disease is endemic. There were 59 cases, with an average annual incidence of 0.05/100,000. The dominant serogroup, *Leptospira icterohemorrhagica*, occurred in 29% of patients; in an earlier study (1970-1979), it accounted for only 2%. Serogroups that occurred mainly in rural areas accounted previously for 79% but had declined to 32%.

Leptospirosis, a zoonotic disease caused by a spirochete, is often related to occupation. Humans are infected through contact with animal reservoirs or a contaminated environment (soil, sewage, or water). Several animal species (rats, mice, or hedgehogs) are natural reservoirs of the disease, while humans are a dead-end host. *Leptospira icterohemorrhagica* is transmitted by rats and is found in sewage water. *L. canicola*, which is usually transmitted by dogs but rarely by cattle and swine, is common among field and irrigation workers. *L. hardjo*, which is transmitted mainly by cattle, is commonly found in dairy workers (1). The clinical spectrum of the disease depends on the serogroup and the host, ranging from a mild flulike illness to severe disease with multiple organ failure (Weil's disease).

Since 1950, Israel has been considered endemic for leptospirosis (1,2) with a peak incidence of 3.6/100,000 in the 1960s (3). From 1970 to 1979, 251 cases of leptospirosis were diagnosed, with a reported attack rate of 0.2/100,000, mainly in agricultural areas (1). Since 1979, no epidemiologic study has reported the prevalence of different serogroups and the epidemiologic pattern of the disease. We have reviewed all known cases of leptospirosis in Israel from 1985 through 1999.

## The Study

In Israel, leptospirosis is a notifiable disease, and reported cases are investigated by the Department of Epidemiology of the Ministry of Health (MOH). For each case, a brief medical report was submitted to the MOH, and an epidemiologic investigation was carried out by an epidemiologic nurse. We reviewed charts at the MOH and serologic information at the central laboratory and extracted data including serologic results, demographic information, residential area, occupation, risk factors, and outcome.

Cases were considered related to occupation if patients were farmers, veterinary doctors, sewage workers, or cattle or swine workers, all occupations known to be associated with leptospirosis. Cases were considered "inner-city related" if the patient had no obvious occupation or activity known to be a risk factor for infection. Residential area was defined as urban or rural.

### Diagnosis

All serologic investigations were done by the microscopic agglutination test. Twenty-two reference serovars of living spirochetes were used, with 20 pathogenic (*L. interrogans)* and two nonpathogenic (*L. biflexa)* serovars.[1] A laboratory-confirmed case was defined as a fourfold increase in antibody titer or a single titer $\geq$1:200.

During the study period, 1985-1999, 59 cases of leptospirosis were serologically confirmed (60% based on the first single serum and the rest on paired sera). Ages of these patients ranged from 16 to 66 years (mean 42 ± 15 years); 53 (90%) were male. Cases occurred throughout the year with no clear seasonality.

The dominant serovars were *L. icterohemhorragica* with 17 cases, followed by *L. hardjo* (12 cases) and *L. balum* (12 cases). The disease was related to occupation in 28 cases, mostly in farmers, including pig farmers and dairy workers. In 19 cases exposure was in the inner city, usually in markets (Table). Most of these cases (13 of 19) were due to *L. icterohemorrhagica*, and the patients were either shopkeepers or occasional shoppers in the markets. The rest of *L. icterohemorrhagica* cases were also from an urban setting, mainly Tel Aviv, but these affected sewage workers, a known risk for leptospirosis. *L. habdomadis* group (serovars *hardjo* and *swajisak*) and *L. gripotyphosa* affected mainly farmers. Information about exposures was not available for eight patients. In our series, one case of *L. icterohemorrhagica*

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[1]*Leptospira* serovars tested: Serovars of *L. interrogans*: Ictero copenhagi Weinberg, Javanica Vcldrat–ATCC 233479, Canicola Hond Utrecht IV-ATCC 2347, Australis–ATCC 23605, Grippothyphosa Moskow V-ATCC 23469, Cynopteri Canazone, Sejroe M-84, Pyrogenes–ATCC 23480, Szwajizak Szwajizak, Ballum Castelloni–ATCC 23580, Mini Sari, Burgas, Hardjo, Ballum Mus, Pomona-ATCC 23478, Tarassovi–ATCC 23481, Bataviae ATCC, Sejreo Bratislava, Rachmat-ATCC 23603, Ictero RGA –ATCC 43642
Serovars of *L. biflexa*: Patoc, Andamana

Table. Characteristics of patients with leptospirosis

| Serovar | No. of cases | Sex | | Living area | | Source of Infection | | Occupation[a] |
|---|---|---|---|---|---|---|---|---|
| | | M | F | Urban | Rural | Inner-city | Occupation | |
| *Leptospira icterohemorrhagica* | 17 | 16 | 1 | 17 | 0 | 13 | 4 | S: 3<br>B: 1 |
| *L. hardjo* | 12 | 11 | 1 | 1 | 10 | 0 | 8 | C: 7<br>B: 1 |
| *L. swajisak* | 4 | 3 | 1 | 2 | 2 | 1 | 1 | S: 1 |
| *L. ballum* | 12 | 10 | 2 | 5 | 7 | 4 | 8 | F: 6<br>S: 2 |
| *L. canicola* | 7 | 7 | 0 | 3 | 4 | 1 | 5 | Sw:4<br>S: 1 |
| *L. gripophytosa* | 3 | 3 | 0 | 0 | 3 | 0 | 2 | F: 1<br>Sw:1 |
| *L. cinopteri* | 1 | 1 | 0 | 1 | 0 | N.A. | N.A. | |
| Mixed | 3 | 2 | 1 | N.A. | N.A. | N.A. | N.A.. | |

[a] Occupations at risk: S = sewage contact; Sw = swine-related occupation; F = ordinary farmers; C = cattle and dairy farmers; B =butcher; NA = information not available.

infection was fatal, for a case-fatality rate of 5.8% among patients infected with *L. icterohemorrhagica.* The clinical manifestations were severe hepatorenal involvement and death after massive cerebral hemorrhage.

Comparison of this period with the earlier report from 1970-1979 (1) shows that serogroups such as *L. habdomadis* and *L. grypotyphosa,* which are associated with farming and had been the dominant pathogens, accounting for 55% and 25% of cases, respectively, had declined to 27% and 5%. The urban serovars of *L. icterohemorrhagica* became the dominant groups, increasing from 2% to 29% (Figure 1).

## Conclusions

Over the last 15 years, several epidemiologic characteristics of leptospirosis in Israel have changed: attack rate, affected population, and dominant pathogenic serogroups. The reported attack rate in Israel has declined from 2 to 3.6/100,000 during 1950-1970, to 0.2/100,000 during the 1980s, and approximately 0.05/100,000 during our study period (Figure 2). This trend is most likely due to improved sanitation and increased awareness of risk factors for the disease. Although underreporting and underdiagnosis cannot be ruled out, the ratio between total serologic tests requested for leptospirosis and the rate of positive results during the last 15 years is extremely low: 1.4% compared with 8% during the 5 years before our study. These data may indicate a higher awareness of leptospirosis among physicians in Israel and do not suggest underdiagnosis.

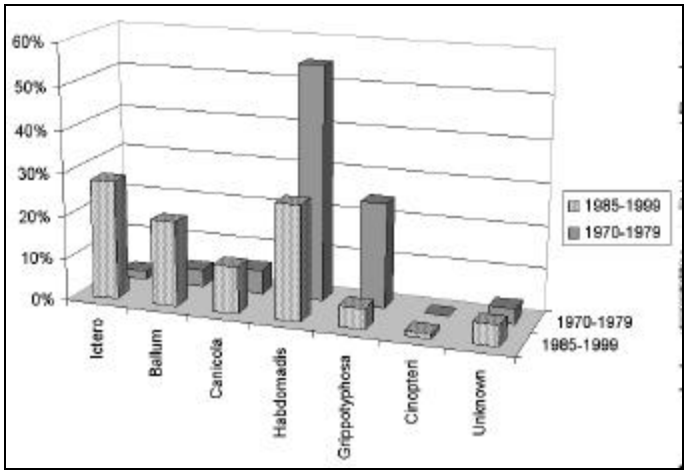


Figure 1. Comparison of *Leptospira* serogroups in Israel

The disease was once more common in rural and agricultural areas and was related to farming. The last report, from 1970 to 1979, showed almost all cases to be rural, while during our study period most cases were urban (mostly in Tel Aviv).

The environmental changes in Israel were associated with a marked change in the epidemiology of pathogenic serogroups. The incidence of the *L. habdomadis* serogroup was 25 cases per year (1), but declined to <1 case per year in our study period (Table). The vectors associated with these groups are cattle and rodents, and therefore farmers and agriculture workers were affected. A recent study in a farming area in Israel where cattle were found to be infected with *L. habdomadis* showed that all 50 farmers in the area who were working with infected animals were seronegative for leptospirosis (A. Barnea, unpub. data). These data may support the assumption that awareness of the disease among these high-risk populations has increased, leading to the use of gloves while in contact with animals. The change to mechanization of field cultivation also prevents contact with animal excreta and thus may reduce leptospira infection.

Over the last few decades, Israel's population has grown rapidly (due to massive immigration), and a trend toward rapid urbanization may also have shifted the disease to the cities. In urban areas, *L. icterohemorrhagica* is the dominant pathogen that causes multisystem involvement (Weil's disease) with a high reported case-fatality rate. This was the main infecting serogroup during our study period, accounting for 29% of all cases, compared with 2% during the previous study. Nonetheless, the absolute number of infected subjects did not change substantially over the last 50 years: During the period 1948 to 1968, there were a dozen cases (2); from 1968 to 1982 there were 14 cases (2); and our study (1984-1999) identified an additional 17 cases. All

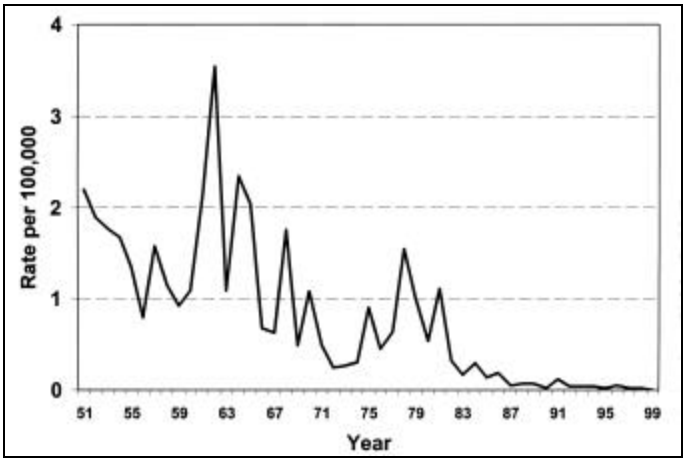


Figure 2. Incidence (rate per 100,000) of leptospirosis in Israel from 1951 to 1999 (adapted from ref. 3, with permission).

*L.icterohemorrhagica* cases were in an urban area, mainly in Tel Aviv, affecting workers in the city's largest market. The vector associated with transmission of *L. icterohemorrhagica* is *Rattus norvegicus*. The last survey of rats in these areas, in 1982, revealed an infection rate of 37% (4), indicating a need for better sanitation control.

There are almost no large-scale reports on the epidemiologic characteristics of the disease in industrialized countries: a report from Ireland during 1990-1996 revealed an annual incidence of 1.2 cases/100,000 (5). In the United States, the annual rate from 1988 to 1994 was approximately 0.02/100,000 (6). Two recent reports from Europe have shown a shift in the epidemiology of the disease, from being an occupational disease towards a disease associated with recreational activities, including travel to tropical countries (7,8). Our case series included only one patient in whom we suspected that the disease was imported (Thailand).

The main change in pattern of the disease in Israel was the decline of occupational-agricultural-related disease and persistence of foci in large cities. Inner-city foci causing sporadic urban leptospirosis have also been described in the United States (9), with *L. icterohemorrhagica* the dominant pathogen. A recent report from Brazil described a large urban epidemic, mainly of *L. icterohemorrhagica* (90% of cases), with a case-fatality rate of 15%, despite aggressive intervention (10). In Brazil leptospirosis had been a sporadic rural disease, but with urbanization and population growth a new environment for urban transmission has been created, mainly in slums and areas lacking proper sanitation (10). Israel exemplifies a rapidly developing country in which urbanization is replacing agricultural areas. Rapid development may allow the formation of foci where adequate sanitation is lacking, such as in markets. More aggressive intervention and vigilance by public health authorities to decrease the rat population in urban areas are warranted.

## Acknowledgments

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Dr. Kariv is a specialist in internal medicine, completing a fellowship in gastroenterology and hepatology at Chaim Sheba Medical Center.

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# The Changing Epidemiology of Malaria in Minnesota

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Malaria cases reported to the Minnesota Department of Health increased from 5 in 1988 to 76 in 1998, paralleling the number of immigrants to Minnesota. In 20% of cases, the *Plasmodium* species was not identified; 44% of cases were hospitalized. The public health community needs to reevaluate current recommendations for refugee screening, provider and patient education, and laboratory capacity.

Malaria infects 300 to 500 million people worldwide and accounts for over 1 million deaths annually (1). Of all infectious diseases, it is second only to tuberculosis in the number of people killed (1). As a result of political unrest and economic hardship, many refugees and immigrants from malaria-endemic areas are moving to nonendemic countries. This provides unique challenges to health-care providers, who may be confronted with diseases not previously observed during their training or clinical practice.

This study examined the changing epidemiology of imported malaria, i.e., malaria that is acquired abroad but diagnosed in the United States. Our goal was to add to previous knowledge of imported malaria by summarizing surveillance data from 1988 through 1998 and by discussing the implications for refugee screening, provider and patient education, and laboratory capacity.

## The Study

Malaria is a reportable disease to the Minnesota Department of Health (MDH) and the Centers for Disease Control and Prevention. All cases of malaria reported from January 1988 to December 1998 were included in this study. A confirmed case was one diagnosed by microscopy in a Minnesota resident with *Plasmodium* species. When a case was reported, a standardized malaria case surveillance form was completed by telephone in consultation with medical providers, laboratory staff, and the patient. Data collected include demographic information, clinical history, travel and immigration history, history of prior malarial infections, and species of *Plasmodium*.

Cases of malaria were categorized as follows: cases in travelers or immigrants, U.S. born or foreign born persons, and U.S. citizen or non-U.S. citizen. A traveler was defined as a person whose travel originated and ended in Minnesota; an immigrant was defined as a person whose travel originated in a foreign country. Citizenship was based on the reported status at the time of diagnosis.

From January 1, 1988, to December 31, 1998, 265 cases of malaria were reported to the MDH. The number of cases per year ranged from a low of 5 cases in 1988 to 76 cases in 1998 (Figure). Demographic characteristics of cases are presented in the Table. Of the 212 cases with reported travel status, 138 (65%) were considered travelers from Minnesota, and 74 (35%) cases were immigrants to Minnesota. From 1988 through 1994, the percentage of cases in travelers was as high as 94%. In 1995 the ratio of travelers to immigrants began to change. By 1998 there were 38 (54%) immigrants and 32 (46%) travelers in the 70 cases with a known status (chi square for linear trend = 15.0; $p<0.005$).

Among those with known citizenship status (n=164), U.S. citizens accounted for up to 78% of the cases per year in the period from 1988 through 1994. By 1998, 18 (28%) of 65 malaria cases (11 cases had an unknown status) had U.S. citizenship, while 47 (72%) were non-U.S. citizens (chi square test for linear trend = 13.5; $p<0.005$).

In 1998, excluding 10 cases for which we could not ascertain birthplace, 13 (20%) cases were born in the United States and 53 (80%) abroad. Of those born abroad with a known country of birth, 33 (87%) were born in Africa, 20 (53%) of these in Liberia (West Africa). Among the 8 patients who were born abroad, sites of malarial infection were Africa and Asia. Most patients typically traveled to or originated as immigrants from West-Central Africa or the Greater Horn of Africa. Liberia (n=29, 55%), the Ivory Coast (n=9, 17%), Kenya (n=6, 11%), Ethiopia (n=4, 8%), and Nigeria (n=4, 8%) were the most common countries where exposure to malaria likely occurred. For the 13 patients in 1998 who were born in

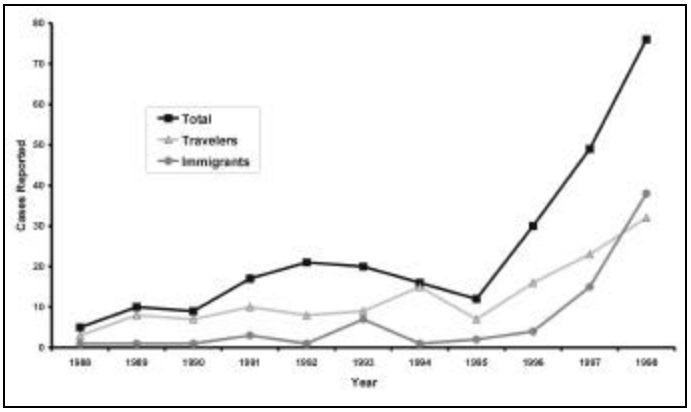


Figure. Reported cases of malaria by year, Minnesota, 1988-1998.

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Table. Demographic characteristics of malaria cases, Minnesota, 1988-1998 (n=265)

| Characteristic | No. of respondents | n | %[a] |
|---|---|---|---|
| Sex | 265 | | |
| Male | | 180 | 67.9 |
| Female | | 85 | 32.1 |
| Residence | 264 | | |
| Twin Cities (seven-county metropolitan area) | | 211 | 79.9 |
| Hennepin County | | 129 | 48.9 |
| Ramsey County | | 63 | 23.9 |
| Age (years) | 260 | | |
| ≤5 | | 26 | 10.0 |
| 6-17 | | 44 | 16.9 |
| 18-29 | | 66 | 25.4 |
| 30-44 | | 76 | 29.2 |
| 45-64 | | 43 | 16.5 |
| ≥65 | | 5 | 1.9 |
| Race | 217 | | |
| Black | | 130 | 59.9 |
| White | | 58 | 26.7 |
| Asian/Pacific Islander | | 23 | 10.6 |
| Hispanic | | 5 | 2.3 |
| American Indian | | 1 | 0.5 |
| Travel origin | 212 | | |
| Traveled from Minnesota | | 138 | 65.1 |
| Immigrated to Minnesota | | 74 | 34.9 |
| Citizenship | 164 | | |
| Non-U.S. citizen | | 95 | 57.9 |
| Primary refugee | | 37 | 22.6 |
| U.S. citizen | | 69 | 42.1 |

[a]Calculations are based on the number of respondents.

the United States, infection occurred in Africa (n=6, 46%), Asia (n=3, 23%), Central America (n=1, 8%), and South America (n=1, 8%). Two patients (15%) were potentially exposed in more than one continent.

Most cases since 1988 were diagnosed with *Plasmodium falciparum* (n=111; 42%), followed by *P. vivax* (n=76; 29%), *P. malariae* (n=14; 5%), and *P. ovale* (n=4; 2%). Laboratory studies of eight cases (3%) showed a mixed infection. *Plasmodium* was identified but no species was determined for 52 (20%) cases. In 1998, there was only 1 U.S.-born case (11%) of *P. falciparum* compared with 25 foreign-born cases (64%). The proportion of U.S.-born cases with *P. vivax* in 1998 was greater than foreign-born cases (odds ratio [OR]=undefined; Fisher's exact 2-tailed test, $p<0.005$). There were two cases of *P. malariae* in 1998; both were in immigrants born in Liberia.

Eleven foreign-born cases from 1998 were asymptomatic and were screened as part of one hospital's refugee assessment. In the period from 1988 through 1998, 109 (44%) of 245 cases were hospitalized for 1 to 30 days (median=3 days). Data from 1998 showed no significant difference in rates of hospitalization between those born in the United States and those born abroad. Complications were reported for 19 (11%) of 170 cases from 1988 through 1998. Data were not available on 95 cases. Two patients were diagnosed with cerebral malaria, 18 with hemolysis or anemia, and 1 with liver failure. No deaths were reported during the 11-year period. No cases of locally acquired or blood transfusion-associated malaria were reported to MDH in 1988 through 1998.

Ninety-nine (52%) of 189 patients indicated that they had a previous history of malaria. In 1998, 37 (59%) of 63 cases had been previously diagnosed with malaria. Patients born outside the United States accounted for 30 (97%) of these malaria cases with a known country of birth.

## Conclusions

We reviewed 265 malaria cases from 1988 through 1998 reported to the MDH. We found that cases, especially among refugees and immigrants, had increased; 20% of cases did not have malarial species identification from blood smears; and 44% were hospitalized. These findings have an impact on current recommendations for refugee screening, provider and patient education, and appropriate laboratory capacity to determine malarial species.

Before the twentieth century, much of the Midwest was endemic for malaria (2). In Minnesota, the last reported cases of locally acquired malaria most likely occurred in the 1930s (3). Since then, there have been major peaks of reported malaria diagnosed in Minnesota that have coincided with the return of soldiers from wars or immigration.

The dramatic climb in cases diagnosed and reported to the MDH in 1997 and 1998 and the increasing proportion of cases among immigrants correspond with increases in primary refugees from Liberia. The number of primary refugees from Liberia climbed from 18 in 1996 to 122 in 1997 and then to 205 in 1998 (MDH, unpub. data). Minnesota was second only to New York in the number of Liberian refugees resettling in the state in fiscal year 1998 (4).

Clinical laboratory training and availability of tests to identify *Plasmodium* at the species level are needed. Species had not been determined for 20% of cases reported to MDH. This has an impact on treatment recommendations because drug resistance and treatment are dependent not only on the country of acquisition but also the species of *Plasmodium*. Diagnosis of malaria is most frequently done by parasite identification on peripheral blood smears. Many laboratory diagnosticians may be uncomfortable diagnosing to the species level (5). Available polymerase chain reaction methods can diagnose malaria at the species level but are limited to reference laboratories (6).

In this study, more than one of every three cases was hospitalized for 1 to 30 days. This may have an impact financially and culturally on immigrants and refugees who have recently arrived in the United States. Many new arrivals may be uninsured or underinsured and have limited or no prior exposure to western medicine.

In 1998, we noted that 11 (14%) of 76 cases were asymptomatic; all were foreign-born immigrants, and 10 (91%) were primary refugees to Minnesota. In Minnesota, initial

health screening for infectious diseases in primary refugees includes tuberculosis, sexually transmitted diseases, hepatitis B, intestinal parasites, and malaria if the person is symptomatic (7). We propose that all primary refugees from malaria-endemic areas be screened for malaria whether or not the person is symptomatic. This would likely prevent future health problems in refugee populations and reduce the risk of autochthonous malaria transmission.

National efforts are needed to support refugee and immigrant health programs that improve access to health care for these populations. Education for health-care providers is needed so that they screen immigrants and refugees appropriately and provide relevant pre-travel advice to those planning return visits to their country of origin. Also needed are culturally sensitive materials for refugees and immigrants that are written in their primary language. This presents unique challenges considering that the diversity of populations resettling in the United States will continue to change, depending on the location of current political and social unrest. The public health community needs to consider these important issues and recommendations as we continue to monitor the influence of immigration on the changing epidemiology of malaria.

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# Reduced Fluoroquinolone Susceptibility in *Salmonella enterica* Serotypes in Travelers Returning from Southeast Asia

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During 1995 to 1999, we collected 1,210 *Salmonella* isolates; 629 were from Finnish travelers returning from abroad. These isolates were tested for susceptibility by determining MICs to ciprofloxacin, nalidixic acid, and seven additional antimicrobial agents. From 1995 to 1999, the annual proportion of reduced ciprofloxacin susceptibility (MIC $\geq$ 0.125 µg/mL) among all travelers' isolates increased from 3.9% to 23.5% ($p<0.001$). The increasing trend was outstanding among the isolates from Southeast Asia; isolates from Thailand alone increased from 5.6% to 50.0% ($p<0.001$). The reduced fluoroquinolone susceptibility was nonclonal in character and significantly associated with multidrug resistance. A point mutation in the quinolone resistance-determining region of *gyrA* was present in all isolates with reduced susceptibility. These data provide further evidence for the rapid spread of multidrug-resistant pathogens from one continent to another.

Fluoroquinolones are active drugs against isolates of the *Salmonella* species (1). There are several reports, however, of treatment failures when these antimicrobials have been used to treat *Salmonella* infections caused by strains with reduced fluoroquinolone susceptibility (2-11). Some epidemiologic studies have shown that the number of *Salmonella* isolates with reduced fluoroquinolone susceptibility has increased, especially in Europe (12,13). Of particular note is the emergence of quinolone resistance in some clones of the widespread *Salmonella enterica* serotype Typhimurium definitive phage type 104 (hereafter *S.* Typhimurium DT104) (14,15). For example, Mølbak et al. (14) recently reported an outbreak caused by a quinolone-resistant *S.* Typhimurium DT104 clone that affected 27 patients in Denmark.

Our preliminary report on fluoroquinolone susceptibility of *Salmonella* isolates in Finland showed an increasing trend in quinolone resistance among isolates classified as being of foreign origin (16). Our study was performed to continue the survey of quinolone resistance and multidrug resistance among isolates of *S. enterica* serotypes. We collected and analyzed *Salmonella* isolates from Finnish patients who acquired the disease either at home or abroad during 1995 to 1999. Special attention was given to delineating the countries and areas associated with reduced fluoroquinolone susceptibility in salmonellae from travelers.

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## Methods

### *Salmonella* Isolates

In Finland, nearly all *Salmonella* isolates recovered from humans (annually 2,500 to 3,500) are sent to the National Salmonella Reference Centre of the National Public Health Institute for typing. In January 1995, a survey was started to monitor antimicrobial resistance in *Salmonella* isolates sent to the Reference Centre. During 1995 to 1999, we collected from this material a total of 1,210 *Salmonella* isolates, with the aim of including only epidemiologically unrelated strains. The possible relationship between different isolates was judged on the basis of epidemiologic information collected from the form that accompanied each isolate. Isolates recovered from distinct sources were determined to be epidemiologically unrelated.

*Salmonella* isolates were divided into two groups according to origin of infection. An isolate was designated to be from a traveler (i.e., foreign), if the patient had reported travel abroad during 1 month before the specimen date. All other isolates were designated to be of domestic (i.e., Finnish) origin. Isolates were collected in five phases: starting in January 1995, we consecutively collected 100 foreign and 100 domestic isolates; starting in September 1996, 200 foreign and 200 domestic isolates; and starting in January 1997, in January 1998, and in January 1999, 100 foreign and 100 domestic isolates, respectively.

### Susceptibility Testing

MICs of isolates were determined by the standard plate agar dilution method according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines (17). The

quinolones evaluated were ciprofloxacin and nalidixic acid; the other antimicrobials were ampicillin, cefotaxime, streptomycin, tetracycline, chloramphenicol, trimethoprim, and sulfamethoxazole. Mueller-Hinton II agar (BBL, Becton Dickinson and Co., Cockeysville, MD) was used as the culture media. *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213, *Escherichia coli* ATCC 25922, *E.coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in testing for susceptibility.

The breakpoint value for reduced ciprofloxacin susceptibility was chosen as ≥0.125 µg/mL on the basis of earlier publications (14) and our recent scatterblot analyses, combined with the sequencing data of the quinolone resistance determining region (QRDR) of the *gyrA* gene (18). For other antimicrobials, MIC breakpoints for resistance used were those recommended by NCCLS (17).

Susceptibility data were analyzed by using the WHONET5 computer program (19,20), available from www.who.int/emc/WHONET/.

### Polymerase Chain Reaction and Sequencing

The QRDR of the *gyrA* gene was sequenced from isolates with reduced ciprofloxacin susceptibility, as we reported previously (18).

### Passenger Statistics

Data concerning the numbers of trips from Finland to countries of interest (i.e., countries with the largest numbers of all *Salmonella* isolates or isolates with reduced ciprofloxacin susceptibility) during the study months were received from Statistics Finland (www.stat.fi/).

### Statistical Analysis

Data were summarized with numbers and proportions of *Salmonella* isolates. Differences in these proportions were statistically tested by applying logistic regression analysis in the following way. Differences between years were modeled as a trend over years. Before doing these analyses, the goodness-of-fit of model of trend was tested. Differences between groups, in trends over years, were analyzed by testing interactions in the models. In addition to assessing crude differences between origin of isolates, an adjusted comparison with year as a covariate was done. Differences were quantified by calculating odds ratios and 95% confidence intervals (CI) (21). Differences between *Salmonella* infection rates in travelers returning from various travel destinations were statistically tested with Poisson regression analysis and quantified with infection rates and 95% CI (22); p values <0.05 were interpreted as significant. Statistical computing was performed by using the SAS system for Windows, release 8.00/1999 applying LOGISTIC or GENMOD procedures.

## Results

### Serotype and Origin of *Salmonella* Isolates

Of 1,210 *Salmonella* isolates studied, 629 were collected from persons reporting travel abroad within 1 month before specimen collection; 581 were classified as of domestic origin. Of all isolates, 98% were from stools and 2% were from extraintestinal sources. Ninety different serotypes were identified. Among the salmonellae isolated from travelers, *S.* Enteritidis and *S.* Typhimurium were the most prevalent serotypes, accounting for 36.2% and 7.8% of those isolates, respectively (Table 1). Among the domestic salmonellae, *S.* Typhimurium accounted for 37.3% and *S.* Enteritidis for 17.0% of isolates.

Of the 629 foreign isolates, the country where salmonellosis was acquired was identified for 618 isolates. For the remaining 11 isolates, origin could be traced to the continental level for 9; the origin of 2 isolates remains unknown.

Table 1. Serotype distribution and reduced ciprofloxacin (CIP) susceptibility (MIC ≥0.125 µg/mL) among 1,210 *Salmonella* isolates studied

| | Isolates from Finnish travelers | | Isolates of Finnish origin | | All isolates | |
|---|---|---|---|---|---|---|
| Serotype | No. of isolates (% of total) | % CIP MIC ≥0.125 µg/mL | No. of isolates (% of total) | % CIP MIC ≥0.125 µg/mL | No. of isolates (% of total) | % CIP MIC ≥0.125 µg/mL |
| *S.* Enteritidis | 228 (36.2) | 3.5 | 99 (17) | 0 | 327 (27) | 2.4 |
| *S.* Typhimurium | 49 (7.8) | 22.4 | 217 (37.3) | 1.4 | 266 (22) | 5.3 |
| *S.* Hadar | 34 (5.4) | 32.4 | 14 (2.4) | 21.4 | 48 (4) | 29.2 |
| *S.* Virchow | 27 (4.3) | 37.0 | 13 (2.2) | 15.4 | 40 (3.3) | 30.0 |
| *S.* Montevideo | 22 (3.5) | 0 | 15 (2.6) | 6.7 | 37 (3.1) | 2.7 |
| *S.* Newport | 18 (2.9) | 5.6 | 13 (2.2) | 0 | 31 (2.6) | 3.2 |
| *S.* Braenderup | 18 (2.9) | 0 | 2 (0.3) | 0 | 20 (1.7) | 0 |
| *S.* Infantis | 17 (2.7) | 5.9 | 33 (5.7) | 0 | 50 (4.1) | 2.0 |
| *S.* Anatum | 17 (2.7) | 11.8 | 4 (0.7) | 25.0 | 21 (1.7) | 14.3 |
| *S.* Panama | 11 (1.7) | 9.1 | 4 (0.7) | 0 | 15 (1.2) | 6.7 |
| Other serotypes (N=80) | 188 (29.9) | 11.2 | 167 (28.7) | 1.2 | 355 (29.3) | 6.5 |
| Total | 629 (100) | 10.5 | 581 (100) | 2.1 | 1,210 (100) | 6.4 |

Most isolates classified as foreign were from travelers to Asia and Europe (Table 2). Altogether, the isolates were obtained from travelers to 53 different countries, with Thailand, Spain, and Turkey the most frequent travel destinations (Table 3).

### Fluoroquinolone Susceptibility

Among all 1,210 *Salmonella* isolates, 78 (6.4%) exhibited reduced susceptibility to ciprofloxacin (MIC $\geq$0.125 µg/mL). These less susceptible isolates consisted of 66 isolates from travelers and 12 of domestic origin. From 1995 to 1999, the annual proportion of reduced ciprofloxacin susceptibility increased from 3.9% to 23.5% ($p<0.001$) among foreign isolates, and from 0% to 4.1% ($p = 0.031$) among domestic isolates (Figure, A,B). An increasing trend throughout the study period was confirmed by logistic regression analysis. The difference between isolates from travelers and those of domestic origin was significant, even after adjustment of year trends ($p<0.001$). The trends of these groups were not different ($p = 0.684$). All isolates with reduced ciprofloxacin susceptibility were uniformly resistant to nalidixic acid (MIC $\geq$32 µg/mL). Thus, the terms reduced fluoroquinolone susceptibility and quinolone resistance are used interchangeably hereafter. All these isolates were susceptible to ciprofloxacin according to NCCLS breakpoint recommendation (MIC $\leq$1 µg/mL).

The 78 isolates with reduced ciprofloxacin susceptibility included 19 different serotypes. The most common were *S.* Hadar (17.9% of isolates), *S.* Typhimurium (17.9%), *S.* Virchow (15.4%), and *S.* Enteritidis (10.3%).

### Quinolone Resistance in Travelers

Isolates with reduced ciprofloxacin susceptibility were obtained from travelers returning from 17 countries; most isolates were from Thailand, Israel, and Spain. The geographic distribution of these isolates by continent is shown in Table 2, which also presents the annual numbers of isolates with decreased ciprofloxacin susceptibility. During the study period, increases in quinolone-resistant isolates from Asia, taken as a whole, and from Southeast Asia alone, were statistically significant ($p<0.001$ for both). Among isolates from Thailand, this increase was especially prominent: from 1 (5.6%) of 18 in 1995 to 17 (50.0%) of 34 in 1999 ($p<0.001$) (Figure, C) (Table 4). Based on the estimated numbers of trips from Finland (during the time the isolates were collected) to the five most frequent countries of origin of all foreign *Salmonella* isolates, as well as of those with reduced ciprofloxacin susceptibility, the infection rates by quinolone-resistant *Salmonella* isolates were highest in travelers returning from Thailand and Malaysia: 0.81 and 0.80 infections per 1,000 trips, respectively (Table 5). (Although the total infection rate of salmonellosis was highest [6.7 infections per 1,000 trips] in Tunisia, the infection rate by quinolone-resistant *Salmonella* isolates was zero.) Despite the high proportion (58.3%) of reduced ciprofloxacin susceptibility in the 12 isolates from Israel (Table 3), the risk of acquiring quinolone-resistant salmonellosis was only 0.30 per 1,000 travels to that country (Table 5). Travelers returning from Spain and Estonia had low infection rates by all salmonellae, including the quinolone-resistant strains.

Among all 31 isolates with reduced ciprofloxacin susceptibility from Thailand, 13 different serotypes were identified; the 17 isolates collected during 1999 were divided into 12 serotypes (Table 4). These findings exclude the presence of one single clone or of a few clones.

Table 2. Number and source of 629 *Salmonella* isolates from Finnish travelers and the annual numbers of isolates with reduced ciprofloxacin susceptibility (MIC $\geq$0.125 µg/mL), 1995 to 1999

| | | Isolates with reduced ciprofloxacin susceptibility | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Year[a] | | | | | | |
| Geographic area | All isolates | 1995 (102) | 1996 (216) | 1997 (107) | 1998 (102) | 1999 (102) | Total (% of all isolates) | p value[b] |
| Africa | **86** | **0** | **0** | **2** | **2** | **0** | **4 (4.7)** | **0.144** |
| America | **27** | **0** | **0** | **0** | **0** | **1** | **1 (3.7)** | **0.132** |
| Asia | **292** | **3** | **6** | **8** | **14** | **18** | **49 (16.8)** | **<0.001** |
| Southeast Asia | 147 | 1 | 1 | 6 | 10 | 18 | 36 (24.5) | <0.001 |
| Middle East | 93 | 2 | 5 | 1 | 2 | 0 | 10 (10.8) | 0.706 |
| Other areas | 52 | 0 | 0 | 1 | 2 | 0 | 3 (5.8) | 0.119 |
| Europe | **222** | **1** | **4** | **1** | **0** | **5** | **11 (5.0)** | **0.079** |
| Mediterranean area | 78 | 1 | 2 | 1 | 0 | 1 | 5 (6.4) | 0.801 |
| Canary Islands | 60 | 0 | 1 | 0 | 0 | 3 | 4 (6.7) | 0.147 |
| Other areas | 84 | 0 | 1 | 0 | 0 | 1 | 2 (2.4) | 0.205 |
| Total | 629[c,d] | 4[c] | 10[c] | 11[c] | 17[c,e] | 24[c] | 66[c,e] (10.5) | <0.001 |

[a] The annual numbers of all isolates from Finnish travelers studied in parentheses.
[b] The differences between years were modeled as a trend over years in these analyses. p value shows the statistical significance of this trend.
[c] Sum of the numbers written in bold.
[d] Two isolates of unknown origin included.
[e] One isolate of unknown origin included.

Table 3. List of the most frequent countries of origin of the 629 *Salmonella* isolates from Finnish travelers and percentage of reduced ciprofloxacin (CIP) susceptibility (MIC ≥0.125 µg/mL)

| Country/area | No. of isolates (% of total) | % CIP MIC ≥0.125 µg/mL |
|---|---|---|
| Thailand | 114 (18.1) | 27.2 |
| Spain (incl. Canary Islands) | 88 (14.0) | 6.8 |
| Turkey | 69 (11.0) | 4.3 |
| Tunisia | 37 (5.9) | 0 |
| Estonia | 32 (5.1) | 3.1 |
| Morocco | 27 (4.3) | 7.4 |
| India | 26 (4.1) | 7.7 |
| Greece | 17 (2.7) | 5.9 |
| Indonesia | 16 (2.5) | 6.3 |
| Cyprus | 14 (2.2) | 7.1 |
| Dominican Republic | 14 (2.2) | 7.1 |
| Kenya | 14 (2.2) | 7.1 |
| Sri Lanka | 14 (2.2) | 7.1 |
| Israel | 12 (1.9) | 58.3 |
| Malaysia | 11 (1.7) | 27.3 |
| Russia | 11 (1.7) | 0 |
| Other areas (N=42) | 113 (18.0) | 4.4 |
| Total | 629 (100) | 10.5 |

### Nucleotide Sequence Analysis

A base substitution in the QRDR of *gyrA* at codon 83 or 87 was present in all 78 isolates with reduced ciprofloxacin susceptibility. The sequence data of mutations in part of these isolates have been described elsewhere (18).

### Resistance to Other Antimicrobials

Among all 1,210 *Salmonella* isolates studied, 56 different resistance profiles were detected. These profiles were analyzed separately for the isolates with reduced ciprofloxacin susceptibility and those fully susceptible. As many as 47.4% of the 78 quinolone-resistant isolates had three or more additional resistance properties, whereas only 11.5% of the 1,132 quinolone-susceptible isolates were resistant to three or more antimicrobials ($p<0.001$).

Of all 629 foreign isolates and 581 domestic isolates, 20.3% and 18.1%, respectively, were resistant to tetracycline. Resistance to sulfamethoxazole was 14.0% among the foreign isolates and 17.7% among the domestic isolates. Corresponding figures were 7.9% and 11.5% for ampicillin and 5.6% and 5.3% for trimethoprim. Of all isolates, 16.9% were resistant to streptomycin and 8.8% were resistant to chloramphenicol. There was no resistance to cefotaxime.

## Discussion

Our study shows a dramatic increase in the annual proportion of reduced fluoroquinolone susceptibility (from 3.9% to 23.5%; $p<0.001$) among all foreign *Salmonella* isolates in Finland between 1995 and 1999. The increasing trend was particularly notable among isolates collected from travelers returning from Southeast Asia, especially Thailand. Moreover, 27.2% of all 114 *Salmonella* isolates from Thailand had reduced fluoroquinolone susceptibility; the proportion of reduced susceptibility was equal among the 11 isolates from Malaysia. The common nature of quinolone resistance in Southeast Asia is illustrated also by our passenger data, which revealed that a tourist's risk of acquiring quinolone-resistant salmonellosis was significantly higher in Thailand and Malaysia than in other destinations. These results clearly show that in the era of frequent international connections, microbes may be easily transmitted from one place to another. Correspondingly, factors furthering the emergence and spread of antimicrobial resistance in any country may soon have an impact on resistance of bacterial pathogens, or even of normal human flora, in faraway regions, even different continents. On this basis, the emergence of antimicrobial

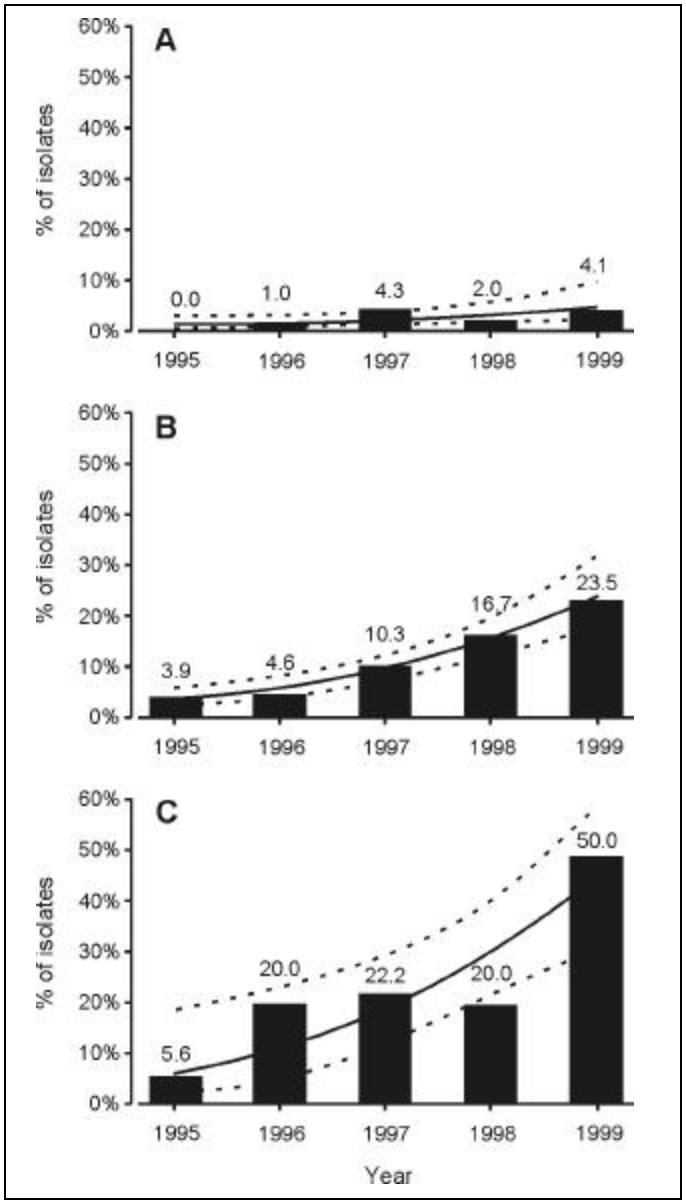


Figure. Percentage of *Salmonella* isolates with reduced ciprofloxacin susceptibility (MIC ≥0.125 µg/mL) of domestic (Finnish) origin (A), from Finnish travelers (B), and from Finnish travelers returning from Thailand (C), according to year. Bars represent observed percentages; the continuous curve represents the predicted trend of logistic model for the percentages; the dashed curves are 95% confidence intervals for the predictions.

Table 4. Serotype distribution of 31 *Salmonella* isolates with reduced ciprofloxacin susceptibility (MIC ≥0.125 µg/mL) from Thailand related to year of isolation

| Serotype | 1995 (N=18/10)[a] | 1996 (N=5/4)[a] | 1997 (N=27/12)[a] | 1998 (N=30/17)[a] | 1999 (N=34/22)[a] | Total (N=114/31)[a] |
|---|---|---|---|---|---|---|
| *S.* Blockley | 1 | | | 1 | 2 | 4 |
| *S.* Haardt | | 1 | | 2[b] | 1 | 4 |
| *S.* Typhimurium | | | 6[b] | | | 6 |
| *S.* Rissen | | | | 2 | 1 | 3 |
| *S.* Hadar | | | | 1 | 3 | 4 |
| *S.* Enteritidis | | | | | 2 | 2 |
| *S.* Virchow | | | | | 2[b] | 2 |
| *S.* Albany | | | | | 1 | 1 |
| *S.* Anatum | | | | | 1 | 1 |
| *S.* Mbandaka | | | | | 1 | 1 |
| *S.* Newport | | | | | 1 | 1 |
| *S.* Panama | | | | | 1 | 1 |
| *S.* Schwarzengrund | | | | | 1 | 1 |
| Total | 1 | 1 | 6 | 6 | 17 | 31 |

[a] Number of all isolates/all serotypes.
[b] Among these isolates, two different resistance patterns were observed.

resistance in any part of the world may have a global bearing and thus deserves universal attention.

When looking for reasons for the rapidly increased quinolone resistance in our travelers' *Salmonella* isolates, three issues must be considered: transferable resistance, mutational resistance, and clonal spread. Until now, transferable resistance to the quinolone antimicrobial group has been described in one preliminary report (23). As far as we know, however, transferable fluoroquinolone resistance appears to be rare in bacteria in vivo. Thus, either clonal spread or resistance due to mutations in chromosomal genes remains the potential mechanism accounting for the high level of reduced fluoroquinolone susceptibility in Southeast Asia. In Thailand, the possibility of clonal spread as a major contributing factor was excluded by identification of 13 serotypes among the quinolone-resistant isolates. In addition, some of these serotypes contained different antimicrobial resistance patterns. Based on these data, we conclude that the reduced fluoroquinolone susceptibility of salmonellae in Thailand primarily involves mutations in chromosomal genes. This concept is consistent with our sequencing data: all 78 *Salmonella* isolates with reduced fluoroquinolone susceptibility (31 from Thailand) so far analyzed in our laboratory have shown a point mutation leading to an amino acid change in their QRDR of the *gyrA* gene. By no means does this finding exclude the presence of any other additional resistance mechanisms.

The emergence of mutation-based resistance may be fostered by selection pressure caused by the use of antimicrobial agents in either human medicine or agriculture. Accordingly, the alarming increase in quinolone resistance observed during the past few years among foodborne pathogens (24-27) has aroused speculation that this might be an effect of the use of quinolones in animal husbandry (14,28). Indeed, two recent articles (29,30) have shown that enrofloxacin (a fluoroquinolone used in agriculture) can select *Salmonella* mutants resistant to nalidixic acid and fluoroquinolones. No part of the world allows quinolones to be used as growth-promoters, but they have been licensed for therapeutic use in food animals in many countries. In Asia, several quinolones, including three fluoroquinolones licensed for humans (ciprofloxacin, ofloxacin, and norfloxacin), have been approved for animal use (31). In Europe, none of the fluoroquinolones licensed for humans are approved for animal use, although many other quinolone preparations are allowed for the treatment of livestock, poultry, and fish. The policy is more strict in the United States, where the only quinolone licensed for food animals is enrofloxacin (32), which is allowed for treatment of poultry alone (31). Without any data on the consumption figures of the quinolone antimicrobial group, no conclusions can be drawn on a potential link between the reduced fluoroquinolone susceptibility of salmonellae and the use of quinolones in animal husbandry in the areas studied. Yet, such a connection is plausible.

On the other hand, the alternative that extensive use of fluoroquinolones in human disease could be responsible for the rapidly increased quinolone resistance of salmonellae seems unlikely, since fluoroquinolones as potent bactericidal drugs are not particularly prone to select for resistance during treatment (33). In direct contrast, treatment with first-generation quinolones (e.g., nalidixic acid) is known to further rapid emergence of resistance in the family of *Enterobacteriaceae* (34,35). Consequently, widespread use of nalidixic acid could easily explain the emergence of reduced fluoroquinolone susceptibility in salmonellae. Again, in the absence of data on the potential use of nalidixic acid for treating salmonellosis in Southeast Asia, such an option can only be hypothesized. The theory is conceivable, however, considering that according to a recent report (36), nalidixic acid is frequently used in Thailand in the treatment of dysentery because resistance of shigella to other antimicrobial groups is common. Another topic of major interest involves

Table 5. Estimated travel-associated *Salmonella* infection rates in Finnish travelers

| Country | Est. no. of trips from Finland during study months[a] | No. of all isolates | Infection rate[b] | Rate ratio[c] | No. of isolates with CIP[d] MIC ≥0.125 µg/mL | Infection rate[b] by isolates with CIP MIC ≥0.125 µg/mL | Rate ratio[c] by isolates with CIP MIC ≥0.125 µg/mL |
|---|---|---|---|---|---|---|---|
| Thailand[e,f] | 38,180 | 114 | 3.0 | 1 | 31 | 0.81 | 1 |
| Spain (incl. Canary Islands)[e,f] | 391,310 | 88 | 0.2 | 0.08 (0.057-0.100) | 6 | 0.02 | 0.02 (0.008-0.045) |
| Turkey[e,f] | 45,427 | 69 | 1.5 | 0.51 (0.377-0.686) | 3 | 0.07 | 0.08 (0.025-0.266) |
| Tunisia[e] | 5,526 | 37 | 6.7 | 2.24 (1.548-3.249) | 0 | 0 | 0 (NA) |
| Estonia[e] | 135,128 | 32 | 0.2 | 0.08 (0.054-0.117) | 1 | 0.01 | 0.01 (0.001-0.067) |
| Israel[f] | 23,014 | 12 | 0.5 | 0.17 (0.096-0.317) | 7 | 0.30 | 0.37 (0.165-0.851) |
| Malaysia[f] | 3,747 | 11 | 2.9 | 0.98 (0.530-1.826) | 3 | 0.80 | 0.99 (0.302-3.225) |

[a]Based on the numbers of Finnish travelers to these countries; data collected from the reports of Statistics Finland.
[b]Infections per 1,000 trips.
[c]Thailand as the reference country. 95% confidence intervals in parentheses. The rate ratios between Thailand or Malaysia and other destinations were significant (p values <0.05).
[d]CIP = ciprofloxacin.
[e]Five most frequent countries of origin of all *Salmonella* isolates.
[f]Five most frequent countries of origin of *Salmonella* isolates with reduced ciprofloxacin susceptibility (MIC ≥0.125 µg/mL).

the potential influence of antimicrobial use in travelers for infections with quinolone-resistant *Salmonella* strains. Unfortunately, data on prophylactic or therapeutic use of antimicrobials were not collected here.

An increasing incidence of reduced fluoroquinolone susceptibility in *S. enterica* serotypes also became manifest in Europe during the 1990s. In England and Wales, reduced ciprofloxacin susceptibility (MIC ≥0.25 µg/mL) in salmonellae increased from 0.3% to 2.1% during the period 1991 to 1994, affecting primarily *S.* Hadar and *S.* Virchow serotypes (12). Concurrently, reduced ciprofloxacin susceptibility emerged in the multidrug-resistant clone of *S.* Typhimurium DT104, of which 1% in 1994 and 6% in 1995 were quinolone resistant (15). There are few U.S. reports of quinolone-resistant salmonellae. Only 21 (0.5%) of the 4,008 U.S. *Salmonella* isolates collected and analyzed during the years 1994 to 1995 were resistant to nalidixic acid (37). Even among the multidrug-resistant *S.* Typhimurium DT104, quinolone resistance in the United States has remained rare (24). However, strains with reduced fluoroquinolone susceptibility are currently not identified in any microbiologic laboratory worldwide according to current NCCLS recommendations, with MIC ≥4 µg/mL of ciprofloxacin as a breakpoint for resistance (17). These breakpoint values are considered adequate, as the clinical importance of the reduced fluoroquinolone susceptibility of salmonellae remains unproven. Nevertheless, we recommend that laboratories worldwide aim at recognizing these less susceptible strains, to reveal their eventual clinical impact. We suggest that laboratories use the nalidixic acid screening test (18) or the E-test to aid identification.

It is noteworthy that the less susceptible subpopulation has already undergone one point mutation and thus is potentially inclined to a second mutation, which could lead to high-level fluoroquinolone resistance. Admittedly, highly fluoroquinolone-resistant *Salmonella* strains are still extremely rare, and they are usually counterselected in field conditions (30). Even so, one can envision that highly fluoroquinolone-resistant *Salmonella* strains, capable of surviving, will inevitably emerge, if the less susceptible strains become prevalent and quinolone pressure persists. On this account, global surveillance of reduced fluoroquinolone susceptibility of salmonellae is also necessary for epidemiologic reasons.

Simultaneous with the increasing incidence of quinolone resistance in salmonellae, rapid emergence of fluoroquinolone resistance is occurring in other enteric bacteria, especially *Campylobacter* sp. and *E. coli* (25-27,38), a situation that threatens to impede the effectiveness of this antimicrobial group. The significantly more common multidrug resistance observed here among the quinolone-resistant salmonellae compared with the susceptible population (47.4% vs. 11.5%) is also of concern. This finding suggests that the use of fluoroquinolones may select for multidrug resistance among salmonellae and provokes a question of whether the same could happen among other bacterial species. In that case, the likelihood of the emergence of notable pathogens, resistant to fluoroquinolones as well as to other commonly used drugs, will certainly increase if the consumption of fluoroquinolones continues to grow. Collectively, these data indicate that prudent use of the quinolone antimicrobial group is warranted to prevent further development of resistance and to preserve the usefulness of these valuable drugs.

In conclusion, we have shown a dramatic increase in reduced fluoroquinolone susceptibility in salmonellae from travelers returning from Southeast Asia. The reduced susceptibility of salmonellae to the fluoroquinolone group was significantly associated with multidrug resistance. Moreover, all quinolone-resistant *Salmonella* isolates had undergone a point mutation in the QRDR of the *gyrA* gene. In contrast to previous reports on quinolone resistance in a specific clone or in a few *Salmonella* serotypes, the reduced fluoroquinolone susceptibility of our isolates was nonclonal. These data provide further evidence of the rapid spread of multidrug-resistant pathogens from one continent to another. The emergence of antimicrobial resistance in any part of the world may have global implications and is, therefore, of universal concern.

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# The Serologic Response to *Cryptosporidium* in HIV-Infected Persons: Implications for Epidemiologic Research

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Advances in serologic assays for *Cryptosporidium parvum* have made serology an attractive surveillance tool. The sensitivity, specificity, and predictive value of these new assays for surveillance of immunocompromised populations, however, have not been reported. Using stored serum specimens collected for the San Francisco Men's Health Study, we conducted a case-control study with 11 clinically confirmed cases of cryptosporidiosis. Based on assays using a 27-kDa antigen (CP23), the serum specimens from cases had a median response immunoglobulin (Ig) G level following clinical diagnosis (1,334) and a net response (433, change in IgG level from baseline) that were significantly higher than their respective control values (329 and -32, Wilcoxon p value = 0.01). Receiver operator curves estimated a cutoff of 625 U as the optimal sensitivity (0.86 [0.37, 1.0]) and specificity (0.86 [0.37, 1.0]) for predicting *Cryptosporidium* infection. These data suggest that the enzyme-linked immunosorbent assay technique can be an effective epidemiologic tool to monitor *Cryptosporidium* infection in immunocompromised populations.

*Cryptosporidium* oocysts are regularly detected in treated and untreated water and have been associated with both food- and waterborne outbreaks (1,2). Of particular concern are immunocompromised persons, among whom HIV-infected persons represent a large group at risk for cryptosporidiosis (3-8). Cryptosporidiosis in HIV-infected persons may be chronic and is associated with substantial mortality. Recent evidence suggests that, in addition to their protracted course of infection, HIV-infected persons may be at higher risk for acquiring infection (9). The introduction of highly active antiretroviral therapy has decreased the incidence of cryptosporidiosis among HIV-positive persons (10), but there are no data to suggest that the incidence of exposure has been reduced.

In part because of limited surveillance tools, much is still unknown about the natural history of cryptosporidiosis (11). To confirm *Cryptosporidium* infection, stool specimens are often examined by microscopy. For epidemiologic studies, this method is problematic because of the short duration of oocyst excretion, the poor sensitivity of the procedure (12), and the amount of laboratory personnel time needed. Moreover, many physicians are unaware of cryptosporidiosis (13). Therefore, since most laboratories examine stools specifically for *Cryptosporidium* only on physician request (14), cryptosporidiosis is generally underdiagnosed.

Serologic assays provide an alternative to parasitologic methods for monitoring *Cryptosporidium* infections. Although many previous studies have used crude extracts of disrupted oocysts as the antigen in an enzyme-linked immunosorbent assay (ELISA), assays based on detection of antibody responses to specific *Cryptosporidium* antigens by immunoblot are more sensitive and specific. When the immunoblot is used, persons exposed to *Cryptosporidium* in outbreak settings have characteristic responses to 27-kDa (immunoglobulin [Ig] G) and 17-kDa (IgA, IgG) antigens, which are found on the surface of sporozoites, the infective stage of the parasite. In studies of volunteers exposed to *C.parvum*, antibodies directed against these antigens were correlated with lower levels of oocyst excretion and with protection from symptomatic infection (15).

From previous studies measuring responses to crude oocyst antigen, it is clear that HIV-positive persons can make antibody responses to *C. parvum* (16); however, these responses have not been assessed by the newer assay formats. Consequently, little is known about the ability of HIV-positive persons to mount an immune response to defined *C.parvum* antigens. The question remains whether HIV-positive persons can mount an antibody response, and, if so, whether the magnitude of the response is associated with CD4 count. To address these issues, we examined 28 clinically confirmed cases of cryptosporidiosis, assaying IgG responses for the 11 cases in which we had blood samples collected after the date of diagnosis.

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## Methods

### Study Population

The San Francisco Men's Health Study (SFMHS) was a prospective study of the epidemiology and natural history of AIDS in a cohort of 1,034 single men between the age of 25 and 54 years (17). The subjects were recruited by multistage probability sampling and followed from 1984 through 1992. The men were followed every 6 months with an interview, a complete physical examination, and collection of clinical specimens. Serum banks have been maintained in liquid nitrogen since the beginning of the study. The institutional review boards of the University of California, Berkeley, and the Centers for Disease Control and Prevention approved this project.

A person was reported to have had cryptosporidiosis if he answered yes to the following question: "Since we last interviewed you (6 months ago), did a doctor or other medical practitioner tell you that you had cryptosporidiosis?" All diagnoses were based on finding oocysts in stool samples. Although we have no information on the illness status of these cases, it is unlikely that stool samples would have been collected for asymptomatic patients.

Records from the SFMHS were reviewed to identify persons diagnosed with *C. parvum* infection. Of 28, 11 had at least one serum sample collected after the date of diagnosis of cryptosporidiosis. To analyze antibody decay, we excluded persons with chronic cryptosporidiosis infection by excluding those with a CD4 count <200, on the assumption that these persons were at high risk for chronic cryptosporidiosis infection.

A control was defined as a person who 1) never had a clinical diagnosis of cryptosporidiosis while under observation; 2) had a serum sample available within 3 months (based on the date of the blood sample used to measure the IgG response after diagnosis of cryptosporidiosis for a matched case); and 3) had a CD4 count at this blood sample date that was within 50 cells/µL of the case CD4 count. For each case, two controls were randomly selected from all possible controls who met these three criteria.

### Rationale for IgG Analysis

Assays based on the detection of IgA, to date, lack sensitivity. IgM results with crude-antigen ELISAs and with immunoblot have suggested that IgM is directed primarily at carbohydrate epitopes. IgM assays thus tend to be characterized by low signal-to-noise ratios and poor specificity. In addition, IgM responses tend to be short-lived, exacerbating issues related to the sensitivity of detection of antibody responses.

### ELISA

Antibody assays used either a recombinant Cp23 protein or a partially purified native antigen fraction isolated from oocysts by Triton X-114 detergent extraction and were performed as described (18). Briefly, antigens were diluted in 0.1 M Na $HCO_3$ buffer at pH 9.6 to concentrations of 0.2 µg/mL (recombinant Cp23) or 0.28 µg/mL (Triton X-114-extracted antigen) and were used to sensitize 96-well plates overnight at 4°C (50 µL/well; Immunlon 2, Dynatech Industries, McLean, VA). Plates were blocked with phosphate-buffered saline (PBS) (0.85% NaCl and 10 mM $Na_2PO_4$ at pH 7.2) containing 0.3% Tween 20 for 1 hour at 4°C, then washed four times with 0.05% Tween 20/PBS. Unknown sera were diluted 1:50 in 0.05% Tween 20/PBS and loaded in duplicate (50 µL/well). Four blank wells (buffer only), duplicate wells containing three positive control sera, and duplicate wells containing four negative sera were included on each plate. A twofold serial dilution (1:50 to 1:12,800) of a strong positive control was also included on each plate to generate a standard curve. The plates were incubated for 2 hours at room temperature. Bound antibodies were quantified by using a biotinylated mouse monoclonal antibody against human IgG (1:1,000 in 0.05% Tween 20/ PBS) (clone HP6017; Zymed Laboratories, South San Francisco, CA) and alkaline phosphatase-labeled streptavidin (1:500 in 0.05% Tween 20/PBS) (Life Technologies, Rockville, MD) with *p*-nitrophenylphosphate substrate (Sigma Chemical Co., St. Louis, MO) as described (18). Absorbances at 405 nm were measured with a Molecular Devices UVmax kinetic microplate reader (Sunnyvale, CA). Antibody levels of unknown samples were assigned a unit value based on the 9-point positive control standard curve with a four parameter curve fit. The 1:50 dilution of the positive control serum was arbitrarily assigned a value of 6,400 U. Arbitrary unit values were expressed per microliter of serum.

### Statistical Analysis

IgG response measures were reported in arbitrary units based on the standard curve described above. These antibody responses were not normally distributed; consequently, the responses of the cases and controls were compared by using the Wilcoxon rank sum test. Least squares regression was used to examine the temporal degradation of antibody responses. A receiver operator curve (ROC) was constructed to examine the sensitivity and specificity of different cutoff values (joint confidence intervals were based on exact methods).

## Results

Table 1 summarizes antibody responses of cases and controls. When the CP23 antigen was used, the median IgG value of the sample collected after the diagnosis of cryptosporidiosis (1,334 U for the cases) was significantly different from the control samples collected at the same time (329 U) ($p<0.05$). The median net increase in IgG levels between the serum samples collected before and after the diagnosis date for cases (433) was also significantly different from that for controls (-32) ($p<0.05$). The time interval between date of diagnosis of cryptosporidiosis and date of the blood sample was 44 to 369 days. After diagnosis with cryptosporidiosis, the median IgG value, when TX17 antigen was used, was not significantly different for cases (140 U) and controls (56 U); however, the median increase in IgG levels between the blood samples before and after the diagnosis date for the cases (71 U) was significantly different from that for controls (-1 U) ($p<0.05$). IgG responses of cases and controls at enrollment into the SFMHS in 1984 were not significantly different, suggesting that in general IgG levels of cases were not distinguishable from those of controls when not associated

Table 1. Summary of antibody responses for the 11 cases and their respective controls

| | IgG peak value CP23[a] | | ΔIgG CP23[b] | | IgG peak value TX17[a] | | ΔIgG TX17[b] | |
|---|---|---|---|---|---|---|---|---|
| | Cases | Controls | Cases | Controls | Cases | Controls | Cases | Controls |
| Mean | 2,784[c] | 414 | 2,117[c] | -17 | 1,594 | 85 | 1,531 | 5 |
| Max | 12,224 | 973 | 11,247 | 157 | 9,264 | 217 | 9,239 | 69 |
| Min | 102 | 178 | -866 | -144 | 36 | 41 | -5 | -47 |
| Median | 1,334[d] | 329 | 433[d] | -32 | 140 | 56 | 71[d] | -1 |
| S.D. | 3,560 | 237 | 3,531 | 84 | 2,880 | 53 | 2,884 | 36 |
| N | 11 | 22 | 11 | 22 | 11 | 22 | 11 | 22 |

[a]IgG response after the date of clinical cryptosporidiosis diagnosis.
[b]Difference in IgG response between a sample taken before and after date of cryptosporidiosis diagnosis.
[c]Case value is significantly different from control value ($p<0.05$).
[d]Case value is significantly different from control value ($p<0.01$).
IgG = immunoglobulin G; TX17 = Triton antigen; SD = standard deviation.

with a cryptosporidiosis diagnosis. There were some notable exceptions, e.g., one control had IgG levels consistently above 1,000 in four measurements from 1984 to 1992. Detailed information on cases and controls is shown in Table2.

Next, we estimated the optimal cutoff or threshold value to be used as a predictor for whether an IgG response permitted classification of the subject as a case or a control. Based on the seven cases that had an IgG measurement within 200 days of the diagnosis date, Figure 1 shows an ROC curve (plot of false positives vs. true positives). A threshold of 625 U was estimated to maximize both the sensitivity (0.86 [0.37, 1.0]) and specificity (0.86 [0.37, 1.0]) of the data; this threshold was chosen as the value on the curve closest to the upper lefthand corner of the graph. We recalculated the ROC curve using the five cases that had an IgG measurement within 100 days of the diagnosis date, as well as the 10 cases with measurements within 300 days. Both analyses resulted in the same estimate for the optimal cutoff value of 625.

The kinetics of the antibody response to the CP23 antigen for each of the 11 cases are shown in Figure 2. Antibody responses were plotted relative to the cryptosporidiosis diagnosis for the 11 cases (time 0 represents the date of diagnosis). The plots were divided into three panels based on the 625-U threshold estimate. The top panel contains the five cases that had an IgG level <625 before and >625 after clinical diagnosis (Cases 1,2,3,4, and 7). All these cases had 3- to 20-fold increases in IgG levels. The middle panel contains the responses that had values >625 before and after clinical diagnosis (Cases 5,6,8, and 9). More detailed observations of these four cases revealed 1) data for Cases 5 and 9 were

Table 2. Serologic response from enzyme-linked immunosorbent assay, using the CP23 antigen, of the 11 cases that had blood samples after the date of clinical diagnosis, and associated controls

| | Case | | | Control | |
|---|---|---|---|---|---|
| Case # | Before dx IgG (CD4/days)[a] | After dx IgG (CD4/days)[a] | ΔIgG response[b] | IgG peak value[c] | ΔIgG response[d] |
| 1 | 204 (786/102) | 637(500/108) | 433 | 178 | 15 |
| 2 | 118 (259/100) | 2,376 (186/115) | 2,258 | 463 | 36 |
| 3 | 977 (96/134) | 12,224(18/216) | 11,247 | 233 | -70 |
| 4 | 342 (248/138) | 5,724 (440/44) | 5,382 | 263 | -22 |
| 5 | 1,084 (1,003/907) | 1,309 (M/47) | 225 | 359 | 79 |
| 6 | 705 (296/71) | 672 (487/369) | -33 | 414 | -80 |
| 7 | 611 (148/202) | 3,911 (52/293) | 3,300 | 605 | -85 |
| 8 | 2,200 (39/114) | 1,334 (33/73) | -866 | 973 | -32 |
| 9 | 891 (159/363) | 2,108 (26/34) | 1,217 | 292 | -35 |
| 10 | 105 (108/79) | 224 (13/92) | 119 | 187 | -144 |
| 11 | 102 (435/132) | 102 (472/238) | 0 | 597 | 157 |

[a]CD4 count and days from cryptosporidiosis diagnosis date.
[b]Difference in IgG response between a sample taken before and after date of cryptosporidiosis diagnosis.
[c]Average of the two controls (CD4 count and sample date were matched to the respective cases).
[d]Average difference in IgG response between the two controls.
M = missing data; IgG = immunoglobulin G; dx = diagnosis.

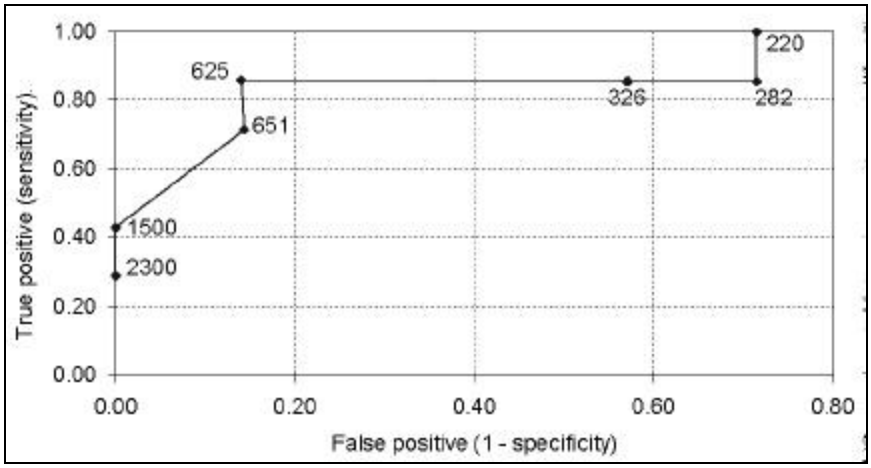


Figure 1. Receiver operator curve for various immunoglobulin G (IgG) cutoff values, based on the seven cases that had a blood sample within 200 days of their diagnosis date.

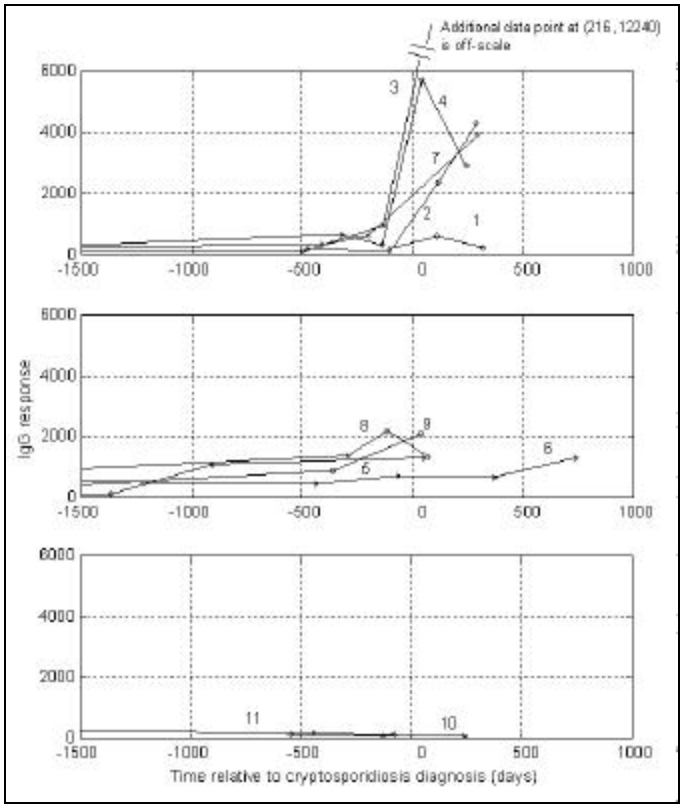


Figure 2. Temporal immunoglobulin G (IgG) response of the 11 cases, grouped by high IgG responders (Cases 1,2,3,4,7), medium responders (Cases 5,6,8,9), and low responders (Cases 10,11). Time zero for each plot is the time of clinical diagnosis.

insufficient to assess the antibody response to infection since the last serum samples collected before diagnosis date were 3 and 1 years before infection, respectively; 2) the lack of response from Case 6 may be because the first available sample after diagnosis was obtained 1 year after diagnosis; and 3) a twofold increase in antibody occurred for Case 8 before the clinical diagnosis date. The bottom panel presents the two cases that remained below 625 U. Case 11 had no increase in IgG levels, and Case 10 had a twofold increase but remained below the threshold.

To determine the duration of antibody response, we analyzed the relationship between antibody level and the time interval between infection and sample collection. To reduce the likelihood that chronic infections would interfere with the analysis, we restricted our analysis to persons with CD4 counts >200. Although sample numbers were small (n = 6), this preliminary analysis suggests that CP23 responses decline to baseline approximately 300 days after an initial response of 3,200 U. Further studies will be necessary to confirm this conclusion.

## Discussion

Study of the natural history of cryptosporidiosis has been limited because of the difficulty of collecting data during the acute phase of the disease. Often, incidence rates are too low to make prospective studies feasible. The blood bank from the SFMHS provided a unique opportunity to study the serologic responsiveness of a cohort of HIV-positive homosexual men clinically confirmed with cryptosporidiosis. Two features of this database make it well suited for a serologic study of cryptosporidiosis: 1) blood was sampled regularly at 6-month intervals from 1984 to 1994, and yearly from 1994 to 1997; and 2) reporting of cryptosporidiosis increased because it is an AIDS-defining condition. We identified 28 clinically diagnosed cases; however, only 11 cases had blood samples both before and after the diagnosis date. To a large extent this was because many of the patients died from *Cryptosporidium* infection. Even when restricted to the 11 cases of cryptosporidiosis with specimens available after diagnosis, the analysis clearly demonstrated the ability of the ELISA using CP23 antigen to discriminate cases from matched controls. These results suggest that the ELISA is a viable approach to identifying recently infected HIV-positive persons. If augmented with data on incidence of diarrhea, this approach could be used to provide valuable estimates of the level of asymptomatic *Cryptosporidium* infection.

One concern with a seroprevalence study in an HIV-positive cohort such as homosexual men in San Francisco is that *Cryptosporidium* exposure might be ubiquitous and chronic. Most of the controls, however, had IgG levels that were significantly below case levels, suggesting that antibody levels are not continuously high in HIV-positive persons. In addition, the IgG responses of cases and controls at enrollment into the SFMHS in 1984 were not significantly different, suggesting that cases were not inherently more responsive to *C. parvum* than controls. The fact that the IgG response of the cases after infection differed from that of controls suggests that this cohort either had a low frequency of exposure or a relatively rapid decay of the antibody response. Our preliminary analysis suggests that a response would decay to control levels after approximately 1 year. This result is consistent with those of other studies (19).

Two limitations to this population-level estimate of IgG degradation are 1) our sample size was small, and 2) chronic infection and multiple exposures may interfere with the natural decay of the IgG response. Because of this small sample size, we were not able to address some potentially interesting and relevant issues, such as the relationship between

serologic response and CD4 count. Since this study provides us with criteria for *Cryptosporidium* exposure, our future studies will not be limited to clinically confirmed cases and will therefore be able to obtain greater sample sizes.

With regard to the second limitation, we assumed that chronic infection was a potential problem and therefore excluded from the analyses subjects with a CD4 count <200/µL—that is, low CD4 count was used as a surrogate indicator for high risk for chronic infection (9,20). Although this was an indirect method of removing chronically infected cases, it provided us with a result that was consistent with previous studies of the kinetics of the antibody response to other antigens. Specifically, when an infection is not chronic, antibody levels decay over time.

The results of our study must also be interpreted in light of the fact that there was no information on the magnitude and timing of the exposure to *C. parvum* nor on prior exposures to *C. parvum* for these cases. These complications may explain why we have three patterns of responses (Figure 2): 1) The strong responders (Cases 1,2,3,4,7), who had low initial levels of IgG and who presumably had no or limited prior exposure; 2) The intermediate responders (Cases 5,6,8,9),who had initial antibody levels consistent with prior exposure or possible chronic infection. Three of these cases (5,8, and 9) had CD4 counts <100, suggesting that the presence of a chronic infection was possible; and 3) The nonresponders (Cases 10 and 11), who never produced levels above control values. The reason for this last pattern of response is not clear. Knowledge of exposure dose may help explain some of these differences. There is no evidence from these data that the level of CD4 count explained the magnitude of the humoral response. For example, Case 3, with a CD4 count of 96, had the strongest IgG response; Case 1, with a CD4 count of 500, had a relatively weak response. Factors responsible for determining the magnitude of the antibody response in infected persons have not been defined.

The Triton antigen (TX17) in this study was less useful than the CP23 antigen in distinguishing cases from controls. Although the TX17 could make this distinction based on the net antibody response to infection, these responses were relatively low. Assays based on use of the TX17 antigen have performed well in previous studies of outbreak populations. A possible reason for our results could be that the antibody response to this antigen is shorter lived. Alternatively, immunodeficient persons may not respond fully to this antigen. Neither explanation is well supported by the data. Cases 5 and 9 had low responses, even though blood samples were collected within 47 days of diagnosis, at a time when peak responses would be expected. Likewise, the second explanation is not supported by the data since one of the three strong responders was Case 3, who had a CD4 count of 96.

Results from this serologic study suggest that surveillance activities could be designed using a serologic test based on the CP23 antigen to estimate the number of recent infections of *Cryptosporidium*. Two pieces of information required before this test can be used are the definition of the optimal threshold IgG value that would define an infection event and the definition of a recent infection. ROC (Figure 1) provides both a method to optimize the choice of a threshold value to identify a case, based on the desired specificity and sensitivity, and a definition of recent infection, based on the decay of the antibody response. ROC analyses suggest that a level >625 U indicates an infection occurred within the past 100 to 300 days.

These results suggest that CP23 has important utility in the study of the epidemiology and natural history of cryptosporidiosis in HIV-infected populations. The value of CP23 in studying other potentially immunocompromised populations (such as oncology patients, children, and the elderly) deserves investigation.

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# *rpo*B Gene Mutations in Rifampin-Resistant *Mycobacterium tuberculosis* Identified by Polymerase Chain Reaction Single-Stranded Conformational Polymorphism

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The use of polymerase chain reaction-single-stranded conformational polymorphism (PCR-SSCP) to study *rpo*B gene mutations in rifampin-resistant (RIFr) *Mycobacterium tuberculosis* has yielded contradictory results. To determine the sensitivity of this method, we analyzed 35 RIFr strains and 11 rifampin-susceptible (RIFs) strains, using the DNA sequencing of the core region of *rpo*B for comparison. Of the RIFr, 24 had a PCR-SSCP pattern identical to that of H37Rv; the other 11 had four different patterns. The 11 RIFs had PCR-SSCP patterns identical to that of H37Rv. The sensitivity of the assay was 31.4%; its specificity was 100%. We observed a strong correlation between the degree of resistance and the type of mutation.

In the developed world, tuberculosis (TB), once considered to have been essentially eliminated, has rebounded and is increasingly caused by drug-resistant strains. In developing countries, however, TB has been an unrelenting scourge. Increasing international travel and migration contribute to its widespread dissemination. Consequently, in 1993, the World Health Organization declared TB to be a global emergency (1).

Drug-resistant TB is a widespread phenomenon, with primary isoniazid-resistance rates as high as 32% and primary multidrug resistance close to 15% in the former Soviet Union. In Latin America, primary resistance to isoniazid varies from 1% in Uruguay to 20% in the Dominican Republic, and primary multidrug resistance is as high as 7% in the Dominican Republic and 5% in Argentina (2). In 1995, we reported increasing resistance rates to isoniazid and rifampin, four times higher than previously reported rates for Mexico (3). Since then, several studies have addressed this issue in different settings: urban, semi-urban, and rural areas. The common finding has been a high rate of primary resistance to isoniazid and to the combination of isoniazid and rifampin (4,5). In 2000, a collaborative effort between the Centers for Disease Control and Prevention and the Mexican TB control program reported an 11% rate of primary isoniazid resistance and 2% of primary multidrug resistance (6).

From the public health perspective, the impact of resistance on disease and death has recently been emphasized (7) in settings where HIV is highly prevalent. However, its impact is also high in semi-urban settings without the influence of HIV infection (8). Thus, reliable methods are urgently needed to rapidly detect resistance, particularly to rifampin (a marker for multidrug resistance), without cumbersome traditional methods or use of radioactivity (9).

Several techniques use polymerase chain reaction (PCR)-based strategies to rapidly detect mutations known to confer resistance. One such method is single-stranded conformational polymorphism (SSCP) analysis, which involves amplification by PCR of a segment of the gene encoding for the specific drug target and comparison of PCR products of drug-sensitive and drug- resistant strains by SSCP, in which mutations usually result in an altered pattern (9,10). This technique is relatively simple and was promising initially, but recent studies have questioned its sensitivity and specificity (10). We investigated the usefulness of PCR-SSCP to detect mutations in the *rpo*B gene of *Mycobacterium tuberculosis* strains with a wide range of rifampin resistance and whether specific mutations in this gene are associated with degree of rifampin resistance.

## Methods

### Clinical Isolates

Forty-six clinical isolates of *M. tuberculosis* were included in this study; all isolates were recovered from sputum samples of patients from Mexico City and were fully characterized by conventional methods (11). All strains were resistant to at least one primary antituberculosis agent (isoniazid 0.1 µg/mL, rifampin 2 µg/mL, streptomycin 6 µg/mL,

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or ethambutol 7.5 µg/mL). Thirty-five strains were rifampin resistant (RIFr), and 11 were rifampin sensitive (RIFs). MICs to the primary antituberculosis drugs were determined by the radiometric method (Becton Dickinson, Cockeysville, MD) (12).

### PCR Amplification

Chromosomal DNA was extracted by conventional methods (13). A 157-bp fragment of the *rpo*B gene was amplified by PCR with primers Tb8 (5'TGCACGTCGCGG ACCTCCA3') and Tb9 (5'TCGCCGCGATCAAGGAGT3'). PCR was carried out in 50 µL of a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 100 µM of deoxynucleoside triphosphates (dNTPs), 1U *Taq* polymerase, 10 pmoles of each set of primers, and 10 ng of chromosomal DNA. Samples were then subjected to one cycle at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 8 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels and examined for the presence of the 157-bp band after ethidium bromide staining.

### Screening of SSCP-PCR Products

The SSCP of PCR products was analyzed by electrophoresis with 12% acrylamide gels. In brief, 25 µL of the amplified product was diluted with 100 µL of buffer (0.1% sodium dodecyl sulfate, EDTA 10 mM); 3 µL of this dilution was mixed with 3 µL of loading buffer (95% formamide, 20 mM EDTA, and 0.05% each of bromophenol blue and xylene cyanol). The mixtures were boiled for 2 min, cooled in ice for 5 min, and then loaded on the gel at 40V for 10 h at room temperature. The gels were silver stained and allowed to dry. The drug-susceptible strain H37Rv was run side by side with the clinical isolates as a control for all experiments. Three different PCR products were analyzed five times each.

### DNA Sequencing

A 411-bp fragment of the *rpo*B gene, containing the sequence of the 157-bp *rpo*B fragment, was amplified by PCR using primers TR1 (5' TACGGTCGGCGAGCT GATCC3') and TR2 (5'TACGGCGTTTCGATGAACC3'). PCR was carried out in 25 µL containing 50 mM KCl, 10 mM Tris (pH 8.0), 0.7 mM $MgCl_2$, 100 µM dNTPs, 1U *Taq* polymerase, and 10 ng of DNA template. Samples were then subjected to one cycle at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 8 min to complete the elongation of the PCR intermediate products. These products were characterized by electrophoresis on 2% agarose gels and stained in 0.5 µg/mL of ethidium bromide.

PCR products were sequenced directly on an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer, Foster City, CA). Samples that gave a single band on agarose gels were purified (Wizard PCR Preps, Promega, Madison, WI) to remove excess primers and nucleotides. Sequencing was done with a PRISM dye terminator cycle sequencing kit (Perkin Elmer), following the manufacturer's instructions.

### Statistical Analysis

Sensitivity and specificity of the PCR-SSCP method were determined by using the test for 2X2 contingency tables. Differences in the mean MIC logs among strains with specific mutations were calculated by the two-sample Wilcoxon rank-sum test (Mann-Whitney U test).

## Results

### Rifr Pattern among *M. tuberculosis* Isolates

The 35 RIFr isolates had MIC values as follows: two isolates had an MIC of 2 µg/mL; six of 8 µg/mL; one of 16 µg/mL; one 32 µg/mL; two of 64 µg/mL; two of 128 µg/mL; eight of 256 µg/mL; one of 512 µg/mL; two of 1,024 µg/mL, and ten of 2,048 µg/mL. All 11 rifampin-susceptible isolates had MIC values $\leq$0.5 µg/mL, but all of them were resistant to at least one other primary antituberculosis agent.

### SSCP Analysis

SSCP assays were repeated at least five times with three different amplicons for all isolates with 100% reproducibility. On the basis of the SSCP results, the 35 RIFr isolates were grouped in two main categories: group one, 24 isolates (68.6%) with an SSCP identical to that of the control strain H37Rv, and group two, 11 isolates (31.4%) with an SSCP different from that of H37Rv. The MICs were variable in group one. In group two, four polymorphisms were observed with different MICs (Figure). The 11 RIFs isolates showed an SSCP identical to that of H37Rv. Therefore, the overall sensitivity of the assay was 31.4%, with a specificity of 100%. It was not possible to correlate the MIC values with the polymorphisms because each strain had a different MIC.

### DNA-Sequencing Analysis

No mutations were found in the core region of the *rpo*B gene in the 11 RIFs isolates. All 35 RIFr isolates showed a mutation by sequence analysis. Seven different missense mutations were observed, with all but one detected within the core region. These mutations produced 13 changes in amino acid content (Table). Mutations at specific codons were associated with the level of resistance; significantly higher MICs were observed when point mutations occurred in codon 513 (median MIC 2,048 µg/mL; p=0.001), in codon 526 (median MIC 2048 µg/mL, p=0.002), and in codon 531 (median MIC 256 µg/mL, p=0.002), compared with mutations at codon 516 (median MIC 8 µg/mL). Single strains

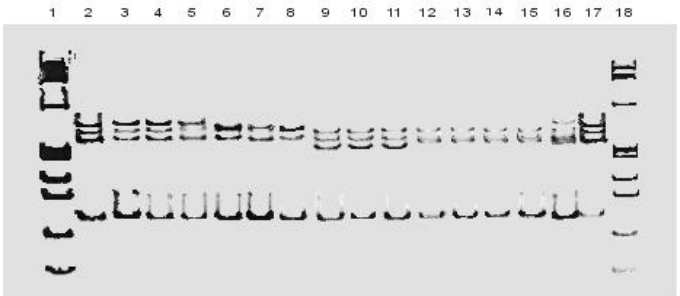


Figure. Representative polymerase chain reaction single-stranded conformational polymorphism (SSCP) patterns of rifampin resistance in *Mycobacterium tuberculosis* strains. Lanes: 1, 18, molecular weight ΦX174/*Hae*III; lanes: 2, 17, *M. tuberculosis* rifampin-susceptible control strain H37Rv; lanes 3, 4, 5 (MICs 2,8,64 µg/mL), pattern 1 rifampin-resistant strains with a pattern indistinguishable from that of *M. tuberculosis* H37Rv; lanes 6,7,8 (MICs 2, 8, 2,048 µg/mL), pattern 2a; lanes 9,10,11 (MICs 2048, 256, 256 µg/mL), pattern 2b; lanes 12,13,14,15 (MICs 2048, 2048, 256, 1,024 µg/mL), pattern 2c; lane 16 (MIC 128 µg/mL), pattern 2d.

with low-level resistance had mutations at codons 522 (MIC 8 µg/mL), 533 (MIC 2 µg/mL), and 572 (MIC 2 µg/mL) (Table). Three mutations in codon 531 had not been described previously. Neither insertions nor deletions were detected in this group of strains.

## Discussion

PCR-SSCP has been used extensively to search for genetic diseases (14,15) and recently to detect missense mutations associated with antibiotic resistance in *M. tuberculosis* (9,10,16,17). In spite of extensive and comprehensive standardization of the PCR-SSCP method, our data show that this procedure was highly specific but had poor sensitivity for detecting mutations in the *rpo*B gene in rifampin-resistant clinical isolates of *M. tuberculosis*, since two thirds of the resistant isolates had a PCR-SSCP pattern similar to that of the *M. tuberculosis* susceptible control strain H37Rv.

Our results differ from those of the investigators who first tested this technique to detect rifampin resistance in *M. tuberculosis* and demonstrated a clear association between *rpo*B mutations and the resistance profile (9). However, in recent studies, this method has detected silent and missense mutations in susceptible strains (18). We did not find these types of mutations, but we did find 24/35 (68.6%) false-negative results. Furthermore, in a recent study by Lee et al. in Korea, false-negative results were obtained in 17 (25.4%) of 67 strains (10). Although suboptimal technical conditions may account for the poor PCR-SSCP performance, missense mutations were found after nucleotide sequencing in rifampin-resistant strains.

Table. Mutations of the *rpo*B gene found in 35 rifampin-resistant *Mycobacterium tuberculosis* isolates

| Mutated *rpo*B codon | Specific Mutation | Strain n | MIC (µg/mL) | p[c] |
|---|---|---|---|---|
| 513 | CAA/AAA(Gln/Lys) | 1 | 2,048 | 0.01 |
| | CAA/AAA(Gln/Lys) | 1 | 256 | 0.01 |
| | CAA/CCA(Gln/Pro) | 1 | 2,048 | 0.01 |
| 516 | GAC/GTC(Asp/Val) | 5 | 37,118 | 0.01, 0.002 |
| 526 | CAC/TAC(His/Tyr) | 1 | 256 | 0.01, 0.002 |
| | CAC/TAC(His/Tyr) | 1 | 1,024 | 0.01, 0.002 |
| | CAC/TAC(His/Tyr) | 3 | 2,048 | 0.01, 0.002 |
| | CAC/GAC(His/Asp) | 2 | 256 | 0.01, 0.002 |
| | CAC/GAC(His/Asp) | 1 | 128 | 0.01, 0.002 |
| | CAC/GAC(His/Asp) | 1 | 2,048 | 0.01, 0.002 |
| 531 | TCG/TTG(Ser/Leu) | 1 | 32 | 0.002 |
| | TCG/TTG(Ser/Leu) | 1 | 64 | 0.002 |
| | TCG/TTG(Ser/Leu) | 4 | 256 | 0.002 |
| | TCG/TTG(Ser/Leu) | 1 | 1,024 | 0.002 |
| | TCG/TTG(Ser/Leu) | 3 | 2,048 | 0.002 |
| | TCG/CCG(Ser/Pro)[b] | 1 | 8 | 0.002 |
| | TCG/GCG(Ser/Ala)[b] | 1 | 64 | 0.002 |
| | TCG/GCG(Ser/Ala)[b] | 1 | 128 | 0.002 |
| | TCG/TGG(Ser/Trp) | 1 | 512 | 0.002 |
| | TCG/TTC (Ser/Phe)[b] | 1 | 2,048 | 0.002 |
| 522 | TCG/TTG (Ser/Leu) | 1 | 8 | |
| 533 | CTG/CCG (Leu/Pro) | 1 | 2 | |
| 572 | ATC/TTC (Ile/Phe)[a] | 1 | 2 | |

[a] This mutation is located outside the core region.
[b] Not previously described.
[c] Mann-Whitney test.

After sequence analysis, all the highly rifampin-resistant (≥128 µg/mL) isolates were found to have point mutations in codons 513, 526, and 531, which were the most common in our study population, corroborating that these mutations are the most prevalent worldwide (19). Since our strains showed different types of mutations, we do not endorse the recently suggested idea of a geographic distribution of single mutations (20,21). Additionally, we observed three alleles in codon 531 that had not been described previously: two strains showed an TCG/GCG (Ser/Ala) exchange, one an TCG/CCG (Ser/Pro) exchange, and another an TCG/TTC (Ser/Phe) exchange.

There was a strong correlation between the degree of resistance and any nucleotide substitution in specific codons. Mutations associated with nucleotide replacements in codons 513, 526, and 531 were associated with high-level rifampin resistance, whereas mutations in codon 516 were observed in low-level rifampin resistance ($p<0.005$) (Table). Other authors have reported high (22,23) and low (24) levels of resistance associated with specific nucleotide replacements. These differences reflect the complex and crucial interaction between the drug and its target at the molecular level, where the position of the affected allele seems to be critical.

An additional interesting finding was that one strain with an MIC of 2 µg/mL did not show a mutation within the 81-bp core region, although a missense mutation ATC/TTC (Ile/Phe) was seen in codon 572, which may explain the rifampin resistance detected in this strain. This observation confirms a recent report of a rifampin-resistant strain identified in Australia with an identical mutation outside the core region of *rpo*B (25).

Although the SSCP method performed poorly in our setting, other DNA sequence-based approaches to detecting rifampin resistance may still have merit, since the vast majority of mutations occur in precisely defined positions. For example, INNO-LIPA (26) is a promising method based on specific detection of previously identified resistance-conferring mutations. Alternatively, other methods based on cell viability, such as use of mycobacteriophages and reporter genes, have shown promise (27, 28).

In conclusion, the practical implications of our study are that the PCR-SSCP method may not be a reliable tool for the detection of resistance to rifampin in *M. tuberculosis*. However,

if our observation of a strong correlation between specific mutations and the level of resistance is confirmed in other settings, the level of rifampin resistance may be predictable by DNA sequence-based resistance detection methods.


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# Detection and Identification of Spotted Fever Group Rickettsiae and *Ehrlichiae* in African Ticks

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*Rickettsia africae*, a recently identified pathogen, was detected for the first time in *Amblyomma* ticks from Niger, Mali, Burundi, and Sudan, and "*R. mongolotimonae*" was identified for the first time in Africa. Rickettsiae of unknown pathogenicity and two new *Ehrlichiae* of the *Ehrlichia canis* group were identified in ticks from Mali and Niger.

Spotted fever group Rickettsiae and *Ehrlichiae* are obligate intracellular gram-negative bacteria associated with arthropods, mainly ticks. While feeding, ticks can transmit these microorganisms to humans and animals (1). Two human tick-borne rickettsioses are known to occur in Africa (2). Mediterranean spotted fever, caused by *Rickettsia conorii*, is transmitted by the brown dog tick, *Rhipicephalus sanguineus*, which is well adapted to urban environments. *R. conorii* is prevalent in the Mediterranean area (Tunisia, Algeria, Morocco, Libya, and Egypt) and has also been isolated or detected in Kenya, Central Africa, Zimbabwe, and South Africa (2). Although African tick bite fever has been recognized since the beginning of the century as a rural disease usually contracted from ticks of cattle and game, it was regarded as synonymous with Mediterranean spotted fever, until the first human infection with *R. africae* was reported from Zimbabwe in 1992. Subsequently, numerous cases have been reported in tourists returning from southern Africa, where the cattle tick *Amblyomma hebraeum* is the vector (2,3). *R. africae* has also been recovered from *A. variegatum* ticks in Ethiopia and central Africa (2). In 1992, a survey for antibodies against *Ehrlichia chaffeensis* (the agent of human monocytic ehrlichiosis) in human sera from eight African countries indicated that human ehrlichioses might occur on the continent (4), and subsequently a case (diagnosed by serology only) was reported from Mali (5). Recently, new molecular methods have enabled the development of useful, sensitive, and rapid tools to detect and identify tick-borne pathogens in arthropods, including ticks (6). In this work, we tested ticks from Africa for rickettsial and ehrlichial DNA using polymerase chain reaction (PCR) and sequence analysis of amplified products.

## Materials and Methods

Ticks were kept frozen at -20°C (in Niger) or at -80°C (in other countries) before being tested. DNA of each tick was extracted as described (7). Rickettsial and ehrlichial DNA was detected by PCR as described, using specific primers (Table).

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The sequences of PCR products were obtained and analyzed with the corresponding sequences of rickettsial or ehrlichial species as described (7). Multiple alignment analysis was performed by using the ClustalW program version 1.8 in the DNA Data Bank of Japan (DDBJ; Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). All sequences used in the study are available in GenBank; the accession numbers of the new genotypes detected in this work are shown in the Table footnotes.

## Results

Rickettsial DNA was detected in 24 (7.2%) of the 332 ticks examined (Table). *R. africae* was detected from *A. variegatum* from Mali (2/6), Niger (2/6), and Burundi (1/13), and from 1 of 16 *A. lepidum* from the Sudan. *R. aeschlimmanii* was detected in *Hyalomma marginatum rufipes* from Niger and Mali (8/24 and 3/20, respectively) and *R. massiliae* in 2/37 *Rh. muhsamae* from Mali. Further, three new *ompA* sequences (590 bp) were obtained from *A. variegatum* from Mali and Niger (Table). These were 99.3%–99.5% identical to those of *R. africae*. In the phylogenetic tree based on these *ompA* sequences, the three rickettsiae (named RAv1, RAv3, RAv9) were closely related to one another (95.7% bootstrap value) and branched with *R. africae* (86.1% bootstrap value) (data not shown). Partial sequences (316 bp) of the *gltA* gene of RAv1, RAv3, and RAv9 were also found to be closely related to those of *R. africae* (99% of similarity). Two new 16S rRNA ehrlichial genotypes were detected, including ERm58 (1,380 bp) in 7/37 *Rh. muhsamae* from Mali and EHt224 (1366 bp) in 1/5 *H. truncatum* from Niger. Both sequences were very similar (99.34% similarity), but different from those described for all the known ehrlichiae (i.e., 98.55% similarity with *E. chaffeensis*, 98.26% with *E. canis* and *E. ewingii*, and 97.75% with *E. muris* and *Cowdria ruminantium*). In a phylogenetic tree based on 16S rRNA gene sequences, ERm58 and EHt224 were found to be closely related and to belong to the *E. canis* group (data not shown). Enlarged *gltA* sequences of ERm58 (1,140 bp) and EHt224 (1,189 bp) were also obtained from the above ticks. Phylogenetic analyses of these sequences confirmed that ERm58 and EHt224 belonged to the *E. canis* group (data not shown).

Table. Detection and identification of spotted fever group *Rickettsiae* and *Ehrlichiae* from African ticks by polymerase chain reaction (PCR)[a,b]

| Tick species | Animal | Location | No. pos. ticks/ total examined | Gene sequence | Identification | GenBank accession no. for new genotypes |
|---|---|---|---|---|---|---|
| *Amblyomma variegatum* | Cattle | Mali | 6/6 (rickettsiae) | ompA | *Rickettsia africae* (2/6) | - |
| | | | | | RAv1 (2/6) genotype | AF311959 |
| | | | | | RAv3 (2/6) genotype | AF311960 |
| | | | | gltA | *R. africae* (2/6) | - |
| | | | | | RAv1 (2/6) genotype | AF311962 |
| | | | | | RAv3 (2/6) genotype | AF311963 |
| | | Niger | 6/6 (rickettsiae) | *ompA* | *R. africae* (2/6) | - |
| | | | | | Rav9 (1/6) genotype | AF311961 |
| | | | | | RAv3 (3/6) genotype | AF311960 |
| | | | | *gltA* | *R. africae* (2/6) | - |
| | | | | | RAv9 (1/6) genotype | AF311964 |
| | | | | | RAv3 (3/6) genotype | AF311963 |
| | | Burundi | 1/13 (rickettsiae) | *ompA + gltA* | *R. africae* | - |
| *A. lepidum* | Cattle | Sudan | 1/16 (rickettsiae) | *ompA + gltA* | *R. africae* | - |
| *Hyalomma impeltatum* | Cattle | Niger | 0/42 | - | - | - |
| | | Mauritania | 0/42 | - | - | - |
| *H. dromedarii* | Cattle | Niger | 0/7 | - | - | - |
| *H. impressum* | Cattle | Niger | 0/8 | - | - | - |
| *H. marginatum rufipes* | Cattle | Niger | 8/24 (rickettsiae) | *ompA + gltA* | *R. aeschlimannii* | - |
| | | Mali | 3/20 (rickettsiae) | *ompA + gltA* | *R. aeschlimannii* | - |
| *H. truncatum* | Cattle | Niger | 1/5 (rickettsiae | *ompA + gltA* | *R. mongolotimonae* | - |
| | | | 1/5 (ehrlichiae) | 16S rRNA | Eht224 genotype | AF311968 |
| | | | | gene | Eht224 genotype | AF311966 |
| | | Mali | 0/5 | gltA | - | - |
| | | Sudan | 0/5 | *ompA + gltA* | - | - |
| | | | | *ompA + gltA* | | |
| *Rhipicephalus muhsamae* | Cattle | Mali | 2/37 (rickettsiae) | *ompA + gltA* | *R. massiliae* | - |
| | | | 7/37 (ehrlichae) | *16S RNA gene* | Erm58 genotype | AF311967 |
| | | | | *gltA* | Erm58 genotype | AF311965 |
| *R. evertsi evertsi* | Cattle | Sudan | 0/10 | - | - | - |
| *R. sanguineus* | Dogs | Mali | 0/24 | - | - | - |
| | | Sudan | 0/62 | - | - | - |

[a] A convenience sample of ticks was obtained as part of other, ongoing studies, as summarized above. In October 1997, 42 *Hyalomma impeltatum* were collected from cattle at Kiffa (16°37'N, 11°24'O) in Mauritania. In Mali in February 1998, 6 *Amblyomma variegatum*, 37 *R. muhsamae*, 20 *H. marginatum rufipes*, and 5 *H. truncatum* were collected from cattle in Bamako (12°39' N, 8°00'W) and Bougouni (11°25' N, 7°29' W) and 24 *R. sanguineus* from dogs in Bamako. In 1999, 6 *A. variegatum*, 42 *H. impeltatum*, 7 *H. dromedarii*, 8 *H. impressum*, 24 *H. marginatum rufipes*, and 5 *H. truncatum* were collected from cattle at Niamey (13°30' N, 2°07' E) in the Republic of Niger; 5 *H. truncatum*, 10 *Rh. evertsi evertsi*, and 16 *A. lepidum* were collected from cattle and 62 *Rh. sanguineus* were collected from dogs in Khartoum (15°31' N, 32°47' E) in the Sudan; 13 *A. variegatum* were collected from cattle in Bujumbura (3°22' S, 29°21' E) in Burundi. All ticks were adults attached on mammals.

[b] Primers include Rr190.70p and Rr190.701n, which amplify a fragment of 629-632 bp of *ompA* encoding for a 190-kD protein (7, and RpCS.877p-RpCS.1273r, which amplify a 396-bp fragment of the citrate synthase gene, *gltA* (7). Ehrlichial DNA was detected with EHR16SR-EHR16SD primers, which amplify a 345-bp fragment of the 16S rRNA gene of all the known ehrlichiae (8). To amplify the main part of the 16S rRNA gene, tick DNA samples that were found to be positive with the above primers were amplified with the EHR16SR and EHR16SD primers and the universal primers fD1 and rp2 (7). The positive DNA samples were also used in PCR reactions to amplify the citrate synthase gene, *gltA*, of *Ehrlichiae.* A sest of primers, EHR-CS133F (5'-GGW-TTY-ATG-TCY-ACT-GCT-GC-3') and EHR-CS778R (5'-GCN-CCM-CCA-TGM-GCT-GG-3'), which amplify a fragment of about 650 bp of the citrate synthase gene of tick-borne *Ehrlichiae*, were used for the screening PCR. Two other primer sets, Chaff-M4F (5'-AAT-TAT-GRT-YAA-ARA-RGC-AG-3')/EHR-CS778R and F1b(5'-GAT-CAT-GAR-CAR-AAT-GCT-TC-3')/Chaff1233R (5'-ACC-AGT-ATA-YAA-YTG-ACG-3') were used to amplify the main part of the citrate synthase gene sequence in tick DNA samples that were found to be positive from the screening PCR (Inokuma, et al., unpub data).

## Conclusion

This study has shown for the first time that *R. africae*, the agent of African tick bite fever, is present in West Africa (Mali and Niger), the Sudan, and Burundi. It also indicates a potential role for *A. variegatum* and *A. lepidum* as vectors of *R. africae* in these areas. Recently, we also documented cases in tourists returning from numerous countries, including those in West and East Africa (9) (Figure). Our results support the hypothesis that the geographic distribution of African tick bite fever parallels that of the distribution of *Amblyomma* spp., as ticks are known to be vectors and also reservoirs of tick-borne rickettsiae (2). In Africa, although the principal vector of *R. africae* appeared to be *A. hebraeum*, which is prevalent in southern Africa, *A. variegatum* (which is widely distributed throughout sub-Saharan Africa) appears as a potential vector. *Amblyomma* are known to readily feed on people in Africa and are commonly infected with rickettsiae (up to 100%). Thus, African tick bite fever may have a high prevalence throughout the continent. Studies have shown seroprevalences of 30%-80% for spotted fever group rickettsiae in sub-Saharan Africa (2), although it is unclear what proportion of those infections might be due to *R. africae* infection.

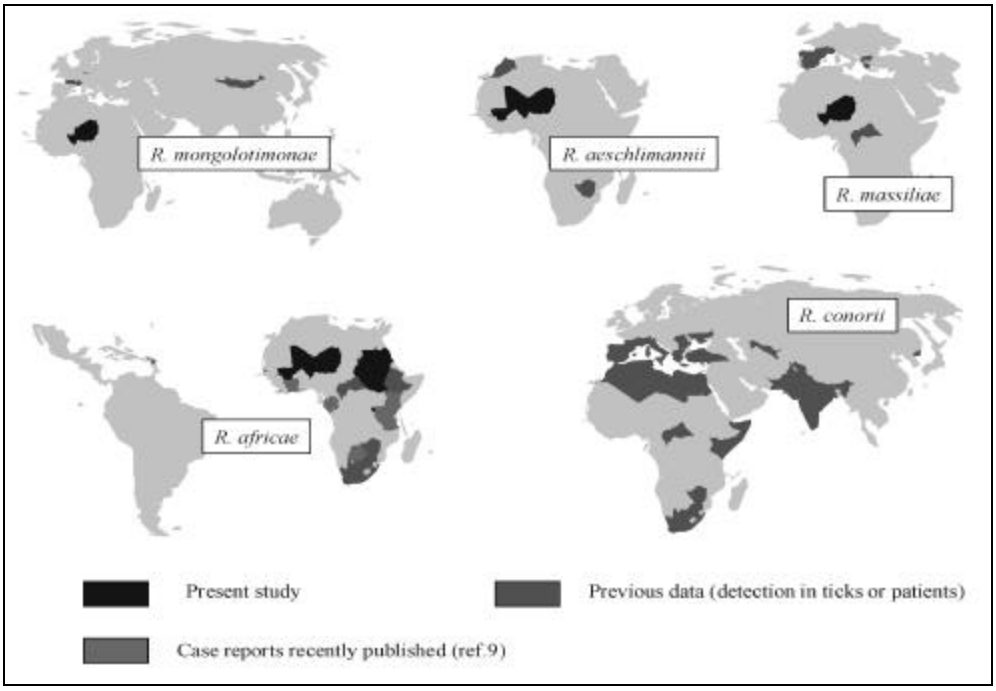


Figure. Geographic distribution of spotted fever group Rickettsiae occurring in Africa. R. = *Rickettsia*.

In this study, we report for the first time the presence of "*R. mongolotimonae*" in Africa (Niger). This pathogen was first isolated from an *H. asiaticum* collected in Inner Mongolia, China. Later, the same agent was isolated from the blood and skin of a febrile woman from Marseille in 1996 (2), which demonstrated its pathogenicity for humans. Subsequently, we have recognized four more cases in southern France (10, and unpub. data). Results of this study suggest that *R. mongolotimonae* may be associated with *Hyalomma* sp. ticks throughout the world. In this work, we also detected two rickettsiae of unknown pathogenicity, namely *R. aeschlimannii* and *R. massiliae*. Although this is the first recognition of these rickettsiae in Mali and Niger, the epidemiologic importance of this finding has yet to be determined. Finally, we detected three new spotted fever group genotypes closely related to *R. africae*. Until further studies clarify the position of these organisms, we suggest they may be considered variant strains of *R. africae*.

Although previous reports, based on the results of serosurveys, have indicated that human ehrlichioses occur in Africa, firm evidence is still absent. Because of the serologic cross-reactivity between ehrlichiae, serosurvey results have to be interpreted carefully. We detected two new ehrlichial genotypes, that is, Erm58 in *Rh. muhsamae* from Mali and Eht224 in *H. truncatum* from Niger. Both belong to the *E. canis* group, which includes *E. chaffeensis*, *C. ruminantium*, *E. muris*, *E. ewingii*, and a new isolate detected in Japanese ticks (11). In this group, as within each group of ehrlichiae, members share homologous surface antigens and thus cross-react extensively in serologic assays (12). Erm58 and Eht224 may also be organisms responsible for such serologic cross-reactions, including in serosurveys and case reports of human ehrlichioses in Africa. In 1997, new ehrlichial genotypes were also detected in Namibia and Zimbabwe, and a number of ehrlichiae in Africa may be responsible for serologic cross-reactions in serosurveys of humans and animals for currently recognized pathogenic ehrlichiae (13,14). The pathogenicity of the Erm58 and Eht224 recognized in our study has yet to be determined, and further studies to characterize the human ehrlichioses in Africa are indicated. Moreover, it remains to be demonstrated whether *H. truncatum* and *Rh. muhsamae* ticks act as vectors or reservoirs of the new ehrlichiae, since ticks also could have been infected while feeding on bacteremic mammals.

Although this study detected for the first time certain richettsiae and ehrlichiae in African countries, systematic sampling was not done, and results cannot address their prevalence and distribution. However, this work provides a starting point for epidemiologic studies there.

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Dr. Philippe Parola obtained both MD and PhD degrees at the Faculty of Medecine of Marseille, France, and is now a postdoctoral fellow at the Harvard School of Public Health, Boston, Massachusetts. His research interests include medical entomology and tick-borne diseases.

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# Vector Competence of Selected North American *Culex* and *Coquillettidia* Mosquitoes for *West Nile Virus*

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To control *West Nile virus* (WNV), it is necessary to know which mosquitoes are able to transmit this virus. Therefore, we evaluated the WNV vector potential of several North American mosquito species. *Culex restuans* and *Cx. salinarius,* two species from which WNV was isolated in New York in 2000, were efficient laboratory vectors. *Cx. quinquefasciatus* and *Cx. nigripalpus* from Florida were competent but only moderately efficient vectors. *Coquillettidia perturbans* was an inefficient laboratory vector. As WNV extends its range, exposure of additional mosquito species may alter its epidemiology.

In 1999, *West Nile virus* (WNV) was recognized for the first time in the Western Hemisphere, causing human, equine, and avian deaths (1-4). Entomologic investigations of this outbreak resulted in the isolation of WNV from two mosquito species, *Aedes vexans* and *Culex pipiens* (2). The distribution of WNV in the United States expanded in 2000 from four northeastern states (Connecticut, Maryland, New Jersey, and New York) to eight additional eastern states (Delaware, Massachusetts, New Hampshire, North Carolina, Pennsylvania, Rhode Island, Vermont, and Virginia) and the District of Columbia (4).

During 2000, evidence of WNV infection was reported in nine additional mosquito species (4). These isolation studies provide preliminary evidence of involvement of several mosquito species in the transmission cycle. However, it is necessary to determine if any of these species are able to transmit WNV by bite before they can be implicated as vectors. In addition, the population density, host preference, feeding behavior, longevity, and seasonal activity of each mosquito species must be considered in determining its relative importance.

In Africa, southern Europe, and western Asia, WNV has been enzootic for many years, with isolations from >40 mosquito species, most in the genus *Culex* (5,6). Laboratory studies indicate that many *Culex* and *Aedes* species in the traditional enzootic range of WNV are competent laboratory vectors (5,6). However, because the introduction of WNV to the United States was recent, little is known about the potential for North American mosquito species to act as vectors of this virus.

Preliminary studies with North American mosquitoes indicate that New York strains of *Cx. pipiens* and *Ae. vexans* are competent but only moderately efficient laboratory vectors (7). The vector competence of *Ae. aegypti, Ae. albopictus, Ochlerotatus atropalpus, Oc. j. japonicus, Oc. sollicitans,* and *Oc. taeniorhynchus* for WNV has since been evaluated (8,9). WNV was isolated from *Cx. restuans* and *Cx. salinarius* caught during the 2000 outbreak in New York (4); however, the ability of these species to transmit WNV by bite is unknown. Other viruses circulating in the eastern United States have a similar epidemiology (e.g., *St. Louis encephalitis* [SLE] and *eastern equine encephalomyelitis* [EEE] viruses): they are maintained in an enzootic cycle involving birds as amplifying hosts and ornithophilic mosquitoes as enzootic vectors. Based on their association with these other arboviruses, several mosquito species should be considered potential vectors of WNV, although it has not yet spread to areas where these mosquitoes are found.

To assist public health personnel in assessing the risk that a potential mosquito vector represents for transmission of WNV, we conducted laboratory studies to evaluate the vector competence of *Cx. nigripalpus, Cx. quinquefasciatus, Cx. restuans, Cx. salinarius,* and *Coquillettidia perturbans.*

## Materials and Methods

### Mosquitoes

We tested five mosquito species for susceptibility to WNV (Table 1). *Cx. nigripalpus* was tested because it is the primary vector of SLE virus in Florida (10,11). *Cq. perturbans* is a potential epizootic vector of EEE virus in the eastern United States (12). *Cx. salinarius* has been found naturally infected with WNV (4) and has been implicated as a potential epizootic vector of EEE virus (12). *Cx. quinquefasciatus* has been implicated as a potential enzootic and epizootic vector of SLE virus (13). *Cx. restuans* has been found naturally infected with WNV (4) and may play a secondary role in the transmission and maintenance of SLE virus (14).

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Table 1. Mosquito species tested for susceptibility to infection with *West Nile virus*

| *Species* | Strain | Source (year collected) | Generation |
|---|---|---|---|
| *Culex nigripalpus* | Indian River | Indian River, FL (2000) | $F_{0-1}$ |
| *Cx. quinquefasciatus* | Sebring | Sebring County, FL (1988) | $>F_{30}$ |
| *Cx. quinquefasciatus* | Vero Beach | Vero Beach, FL (1999) | $F_{10-12}$ |
| *Cx. restuans* | Maryland | Frederick & Prince George's Counties, MD (2000,2001) | $F_0$ |
| *Cx. salinarius* | Chambers | Chambers Co., TX (1992) | $>F_{30}$ |
| *Cq. perturbans* | Laurel | Laurel, MD (2000) | $F_0$ |

### Virus and Virus Assay

The WNV strain (Crow 397-99) used was isolated from a dead crow found in the Bronx, New York, during an epizootic in 1999 (7); it had been passaged once in Vero cell culture. Stocks of virus at a concentration of $10^{4.2}$ PFU/mL were prepared in a standard diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts [GIBCO-BRL, Gaithersburg, MD] $NaHCO_3$, and antibiotics). Viral stocks, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on Vero cells as described (15), except that the second overlay, containing neutral red stain, was added 2 days after the first overlay.

### Vector Competence Studies

Mosquitoes were allowed to feed on 2- to 3-day-old leghorn chickens (*Gallus gallus*) that had been inoculated with approximately $10^3$ PFU of WNV 1 to 2 days earlier. Immediately after the mosquitoes fed, blood was drawn from the jugular vein of each chicken (0.1 mL of blood into 0.9 mL of heparinized diluent), and the blood suspensions were frozen at –70°C until assayed for virus to determine viremias at the time of mosquito feeding. After feeding on viremic chickens, engorged mosquitoes were transferred to 3.8-L screen-topped cardboard cages and held at 26°C with a 16:8(L:D)-hour photoperiod. After an incubation period of 12 to 14 days, the mosquitoes were allowed to feed again on 1- to 2-day-old chickens, either individually or in small groups, to determine if they could transmit virus by bite. Immediately after the transmission attempt, the mosquitoes were killed by freezing, their feeding status was determined, and their legs and bodies were triturated separately in 1 mL of diluent.

Infection was determined by recovery of virus from the mosquito tissue suspension. If virus was recovered from its body but not its legs, the mosquito was considered to have a nondisseminated infection limited to its midgut. If virus was recovered from both the body and leg suspensions, the mosquito was considered to have a disseminated infection (16). We defined the infection and dissemination rates as the percentages of mosquitoes tested that contained virus in their body or legs, respectively. Chickens used in the transmission attempts were bled from the jugular vein 2 days after mosquito feeding, and the blood was handled as described above. Recovery of virus from this blood indicated transmission (9).

To examine viral transmission more efficiently, some of the unfed mosquitoes were inoculated intrathoracically (17) with 0.3 µL of a viral suspension containing $10^{4.2}$ PFU of WNV/mL ($10^{0.7}$ PFU/mosquito), held 7 to 14 days, and allowed to feed on 1- to 2-day-old chickens. Mosquitoes and blood specimens from these chicks were processed as described for the orally exposed mosquitoes.

To estimate transmission rates by species, we determined the percentage of mosquitoes with disseminated infection (after either oral exposure or by intrathoracic inoculation) that transmitted virus by bite. We then multiplied that percentage times the percentage of mosquitoes that developed a disseminated infection after feeding on a host with a particular viremia. The result is the estimated transmission rate for those mosquitoes.

### Statistical Analysis

Confidence intervals (95%) for infection and dissemination rates were calculated by SAS 8.0 (18). We used Fisher exact test to compare transmission rates among disseminated mosquitoes in each species. Significance was tested at a level of alpha = 0.05.

## Results

All mosquito species examined in this study were susceptible to infection with WNV and developed disseminated infections (Table 2). Infection rates were >84% in all the *Culex* species when the viral titer in the donor chicken was $\geq 10^{6.3}$ PFU/mL of blood. In contrast, the infection rate was 18% in *Cq. perturbans* fed on a chicken with a similar level of viremia. For most mosquito species tested, dissemination rates were approximately one fourth the infection rates.

None of the *Culex* species tested differed significantly in the percentages of mosquitoes with disseminated infection that transmitted virus (Table 3). However, the percentage of *Cq. perturbans* with disseminated infection that transmitted WNV was significantly lower than that for *Cx. nigripalpus* and *Cx. quinquefasciatus* (Fisher exact test, $p < 0.01$).

We used the percentage of mosquitoes with disseminated infection that transmitted virus from Table 3 and the dissemination rates at 14 days after the infectious blood meal from Table 2 to estimate the transmission rate for each species. Under laboratory conditions and at the highest viral dose tested, the *Culex* species tested were moderately efficient vectors (estimated transmission rates 10% to 55%). In contrast, *Cq. perturbans* was an inefficient vector (estimated transmission rate $\leq$2%) (Table 2).

## Conclusions

Previous laboratory studies indicate that a number of North American mosquito species could serve as vectors of WNV (7-9). Our study indicated that several additional *Culex* species and *Cq. perturbans* are potential vectors of WNV. The viremias used in our study, $10^{5.5-7.5}$ PFU/mL of blood, are consistent with levels considered to be low to moderate viremias for hooded crows and house sparrows in Egypt (19) and experimentally infected North American house sparrows and other passerine birds (N. Komar, pers.

Table 2. Infection, dissemination and estimated transmission rates for mosquitoes orally exposed to *West Nile virus*

| Species | Strain | Viral dose[a] | No. tested | Infection rate[b] | Dissemination rate[c] | Estimated transmission rate[d] |
|---|---|---|---|---|---|---|
| *Culex nigripalpus* | Indian River | 4.6 | 7 | 29 ([4-71], 2) | 0 ([0-41], 0) | 0 |
| | Indian River | 5.7±0.5 | 132 | 78 ([70-85], 103) | 8 ([4-14], 11) | 7 |
| | Indian River | 6.8±0.4 | 127 | 84 ([77-90], 107) | 12 ([7-19], 15) | 10 |
| *Cx. quinquefasciatus* | Sebring | 5.5 | 16 | 50 ([25-75], 8) | 6 ([0-30], 1) | 6 |
| | Sebring | 7.0±0.5 | 78 | 91 ([82-96], 71) | 22 ([13-33], 17) | 20 |
| | Vero Beach | 5.0 | 13 | 46 ([19-75], 6) | 0 ([0-25], 0) | 0 |
| | Vero Beach | 6.3 | 17 | 94 ([71-100], 16) | 12 ([1-36], 2) | ≤13 |
| *Cx. restuans* | Maryland | 6.6±0.3 | 11 | 100 ([72-100], 11) | 55 ([23-83], 6) | 55 |
| *Cx. salinarius* | Chambers | 6.6±0.3 | 20 | 95 ([75-100], 19) | 60 ([36-81], 12) | 34 |
| *Coquillettidia perturbans* | Laurel | 6.6±0.3 | 11 | 18 ([2-52], 2) | 9 ([0-41], 1) | 2 |

[a]$Log_{10}$ PFU/mL of blood.
[b]Percentage of mosquitoes containing virus in their bodies ([95% confidence interval (CI)], number infected).
[c]Percentage of mosquitoes containing virus in their legs ([95% CI], number disseminated).
[d]The estimated transmission rate = the percentage of mosquitoes that developed disseminated infection 12-14 days after ingesting WNV multiplied by the percentage of mosquitoes with disseminated infection that transmitted virus by bite (Table 3).

comm.). Thus, our results should reflect what would happen when mosquitoes feed on birds circulating a similar concentration of virus in nature.

The *Culex* species tested in this study were moderately efficient vectors, with estimated transmission rates from 10% to 55% after exposure to viremias $\geq 10^{6.3}$. For comparison, the estimated transmission rate for *Cx. pipiens* held under conditions similar to those of our study is 20% (9). Although the *Culex* species tested were readily susceptible to oral infection, most infections were limited to the midgut and did not disseminate to the hemocoel. This finding is similar to results reported for *Cx. pipiens*, in which 81% became infected but only 23% developed disseminated infection (9). Compared with the moderately efficient *Culex* mosquito vectors of WNV, selected container-breeding *Aedes* and *Ochlerotatus* species are highly efficient vectors, and selected floodwater *Aedes* and *Ochlerotatus* mosquitoes are inefficient laboratory vectors (7-9). The *Cq. perturbans* in our study fell into the inefficient vector category.

*Cx. nigripalpus* has not been found naturally infected with WNV. However, the distribution of WNV in the United States is just beginning to reach the southern half of North Carolina, the northernmost limit of these mosquitoes' geographic distribution. *Cx. nigripalpus* is likely to become involved in WNV transmission because it is a primary vector of SLE in Florida (10,11) and is a competent laboratory vector of WNV. Furthermore, *Cx. nigripalpus* is an opportunistic feeder (20,21) and shifts host selection based on the season, feeding on avian hosts in the winter and spring and on mammalian hosts in the summer and fall (22). These factors, coupled with the vector competence data, suggest that *Cx. nigripalpus* could serve as an epizootic as well as an enzootic vector for WNV.

Our study showed that *Cx. quinquefasciatus* can transmit WNV by bite. WNV has not been isolated from wild-caught *Cx. quinquefasciatus*. However, the current distribution of WNV is just beginning to overlap the geographic range of this species (generally the southern United States). *Cx. quinquefasciatus* has been implicated (through virus isolation and abundance during outbreaks) in the rural transmission of SLE virus in the western United States (23) and in urban transmission of SLE virus in the southern United States (24). In contrast to *Cx. pipiens*, which primarily feeds on birds, *Cx. quinquefasciatus* shows a preference for avian blood but will feed readily on mammals, including humans (25). The data from this study, the bionomics of *Cx. quinquefasciatus*, and the mosquitoes' association with an arbovirus with similar epidemiology to WNV, suggest that *Cx. quinquefasciatus* may play a role in WNV transmission if—or more likely when—the distribution of the mosquito and the virus overlap to a sufficient degree.

*Cx. restuans*, which has been found naturally infected with WNV (4), transmitted WNV by bite in our study. Similarly, this species has been implicated as a vector of SLE

Table 3. Percent of mosquitoes with disseminated infection (after either oral exposure to or intrathoracic inoculation with *West Nile virus*) that transmitted virus by bite

| Species (strain) | No. tested | Percent transmission[a] |
|---|---|---|
| *Culex nigripalpus* (Indian River) | 15 | 87 ([60-98],13)a |
| *Cx. quinquefasciatus* (Sebring) | 18 | 94 ([73-100],17)a |
| *Cx. quinquefasciatus* (Vero Beach) | Not determined | |
| *Cx. restuans* (Maryland) | 2 | 100 ([16-100],2) a,b |
| *Cx. salinarius* (Chambers) | 16 | 56 ([30-80],9)a,b |
| *Coquillettidia perturbans* (Laurel) | 17 | 24 ([7-50],4)b |

[a]Percentage of mosquitoes with disseminated infection that transmitted virus by bite (95% confidence interval), number transmitting). Percent transmissions followed by the same letter are not significantly different at alpha = 0.05 by Fisher exact test.

virus by virus isolations from field-collected specimens (26,27), and its role is supported by laboratory transmission studies (13). *Cx. restuans* breeds in ground pools or container habitats, is widespread in its distribution in the United States, and adults are active early (by mid-May) in the eastern United States (28). This early season abundance, along with coinciding isolations of SLE virus from this species in early summer, implies that it may be involved in the overwintering or amplification of SLE virus (26). The isolation of WNV from *Cx. restuans* in July in Connecticut (29), relatively early in the WNV transmission season, raises concern that the role of *Cx. restuans* in WNV transmission may be similar to the one suggested for SLE virus. *Cx. restuans* feeds primarily on avian hosts (30), but whether it feeds on humans remains unclear (31). Given the lack of firm data on host preference, the role of this species as an enzootic or epizootic vector of WNV is still uncertain.

Our study indicated that *Cx. salinarius* transmits WNV efficiently by bite. During 2000, evidence of WNV infection was reported in 35 pools of this species, second in number only to the number of positive pools (126) of *Cx. pipiens* (4). To date, no summary of the data (e.g., minimum infection rates) from the 2000 season has been published, so the relative importance of these isolates cannot be compared. In general, *Cx. salinarius* appears to be mammalophagic in studies of blood meals, but its host feeding pattern is thought to be opportunistic, depending on host availability, innate host preference, or combination of these factors (20,25,32,33). Given the number of WNV-positive pools, its vector competence for WNV, and its feeding behavior, *Cx. salinarius* may be an ideal bridge vector between the enzootic avian cycle of WN and mammalian hosts.

*Cq. perturbans* was the least efficient WNV vector of those we tested. Contributing heavily to this finding was the presence of a salivary gland barrier. Less than one fourth of *Cq. perturbans* with disseminated infection transmitted WNV by bite (Table 3). Furthermore, this is the only North American species tested so far that exhibits a substantial salivary gland barrier. *Cq. perturbans* is generally regarded as mammalophagic (30,34); however, there are reports of its feeding on wading birds and passerines (34-36) and of numerous EEE virus isolates from field-collected specimens (37-40). Despite the low transmission rate, the role of *Cq. perturbans* as a potential epizootic vector of WNV should not be totally discounted.

Our study extended the list of potential North American mosquito vectors of WNV. None of the North American species tested in this study or others (7-9) was refractory to WNV. However, there is a wide range in vector competence in these species, ranging from nearly incompetent (e.g., *Cq. perturbans*) to highly efficient (e.g., *Oc. j. japonicus*). These data are similar to those for Old World mosquito vectors of WNV, in which all *Aedes* and *Culex* species tested were competent vectors (5,6). Vector competence studies indicate that North American mosquitoes fall into three general categories depending on genera and, in some instances, breeding habitat: highly efficient, container-breeding *Aedes* and *Ochlerotatus* species; moderately efficient, *Culex* species; and inefficient, floodwater-breeding *Aedes* and *Ochlerotatus* and *Cq. perturbans*.

As WNV extends its range southward and westward, additional mosquito species (e.g., *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Cx. tarsalis*, and *Ae. albopictus*) will have greater exposure to this virus. Involvement of some of the species, particularly container-breeding *Aedes* and *Ochlerotatus*, may alter the epidemiology of WNV and present additional control problems for mosquito abatement personnel. In addition, mosquitoes are more efficient vectors at warmer temperatures (41,42; Dohm, unpub. data), a factor that will further change the epidemiology of WNV as its range extends southward.

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# Vancomycin-Intermediate *Staphylococcus aureus* in a Home Health-Care Patient

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In June 2000, vancomycin-intermediate *Staphylococcus aureus* (VISA) was isolated from a 27-year-old home health-care patient following a complicated cholecystectomy. Two VISA strains were identified with identical MICs to all antimicrobials tested except oxacillin and with closely related pulsed-field gel electrophoresis types. The patient was treated successfully with antimicrobial therapy, biliary drainage, and reconstruction. Standard precautions in the home health setting appear successful in preventing transmission.

To date, four of the eight cases of infection by *Staphylococcus aureus* with reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus* [VISA] or glycopeptide-intermediate *S. aureus* [GISA]) have been reported in the United States (1-3). We report a fifth case of VISA infection in the United States, and the first to occur during home health-care therapy. While all previous VISA strains have been oxacillin resistant, one of the two VISA strains identified in this investigation was oxacillin susceptible.

## Case Report

The patient, a 27-year-old woman, was well until acute cholecystitis developed in February 2000. She had undergone left nephrectomy and radiation therapy at 4 years of age for Wilms tumor. There was no evidence of tumor recurrence, and she had normal renal function. She worked as a nurse in several long-term care facilities for 6 years.

She underwent emergency laparoscopic cholecystectomy that was complicated by laceration of the common bile duct. She required open cholecystectomy and placement of a biliary drainage tube (i.e., T-tube). Cefotetan (1,000 mg) had been given parenterally as perioperative prophylaxis. A T-tube cholangiogram performed 2 weeks after the procedure showed no common bile duct obstruction or stricture, and the T-tube was removed. During the next 2 weeks, fever and right upper quadrant abdominal pain developed. A computed tomography (CT) scan of the abdomen demonstrated multiple abscesses in right and left hepatic lobes. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrated mild narrowing of the common bile duct. A biliary stent was placed and levofloxacin and metronidazole were administered, but the patient's condition did not improve. Aspiration of one abscess under CT guidance revealed no pathogens. A bile culture obtained during ERCP stent replacement identified *Candida albicans* and oxacillin-resistant *S. aureus* (ORSA, vancomycin MIC=2 µg/mL). Intravenous vancomycin, metronidazole, levofloxacin, and fluconazole were administered, and the patient was transferred from the hospital in Nevada to Los Angeles, where a transhepatic biliary drainage catheter was placed. In April 2000, she was discharged to home, where she was followed weekly by home health-care nurses.

Over the next 10 weeks, the patient received ciprofloxacin (500 mg every 12 hours), metronidazole (250 mg every 8 hours), and vancomycin (from 750 mg to 1,100 mg every day). Serum vancomycin trough levels obtained approximately once each week were from 2.7 µg/mL to 4.9 µg/mL. During elective exchange of the transhepatic biliary drain on June 6, 2000, a culture of bile drainage identified *C. albicans*, *Stenotrophomonas maltophilia*, ORSA, and VISA.

After VISA was confirmed, vancomycin therapy was stopped, and treatment was modified to include linezolid (600 mg twice a day), trimethoprim-sulfamethoxazole (160 mg and 800 mg twice a day), and doxycycline (100 mg orally twice a day). After 10 days of therapy, the patient's condition improved, and repeat culture of the bile showed no growth of *S. aureus*. Six weeks after therapy for VISA was initiated, the patient underwent reconstruction of the bile duct with a Roux-en-Y hepaticojejunostomy and removal of intrahepatic drain. During this hospitalization, therapy for VISA was discontinued. Although the VISA infection cleared, the patient continues to have symptoms of gastroesophageal reflux disease.

After 24 hours' incubation, primary isolation purity plates of biliary drainage grew large, yellow, beta-hemolytic colonies of *S. aureus* with an oxacillin MIC >16 µg/mL and a vancomycin MIC of 2 µg/mL by broth microdilution. After 48 hours of incubation, additional small colonies were visible, and two morphologically distinct strains of *S. aureus* were identified on subculture. In the clinical laboratory, vancomycin susceptibility of both strains of *S. aureus* was determined by broth microdilution (vancomycin MIC = 8 µg/mL) and was confirmed by E-test (vancomycin MIC = 6 µg/mL) and

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growth on a brain heart infusion screening plate containing 6 µg of vancomycin. These tests were repeated and confirmed on both strains at the Centers for Disease Control and Prevention.

The antimicrobial susceptibility profiles of the two strains were identical for 10 of the 11 antimicrobials tested: susceptible to gentamicin, tetracycline, quinupristin-dalfopristin, and trimethoprim-sulfamethoxazole; intermediate to vancomycin and chloramphenicol; and resistant to erythromycin, clindamycin, levofloxacin, ciprofloxacin, and rifampin. However, strain no. 1 was resistant to oxacillin (MIC >16 µg/mL) and was *mecA* positive by polymerase chain reaction (4); strain no. 2 was susceptible to oxacillin (MIC = 0.5 µg/mL) and was *mecA* negative.

Before the identification of VISA, the home health-care agency nurses caring for the patient had routinely used standard precautions (i.e., washing hands before and after contact with the patient, using gloves for contact with nonintact skin or mucous membranes, and wearing mask and eye protection if splashing was anticipated). Because the patient administered vancomycin and emptied the biliary drainage reservoir herself, nurses did not wear face shields or masks before VISA was identified. The nurses' activities generally were limited to inspecting and flushing the percutaneously inserted central venous catheter. After identification of VISA, additional measures were instituted, including care by a single designated nurse and use of gloves, mask, and gowns for all patient contact (5).

Before these precautions were instituted, we assessed possible person-to-person transmission of VISA. Cultures were obtained on dry cotton swabs from both anterior nares of the patient, of her household contacts (parents and spouse), and of the three nurses who had provided care in the previous 3 months. Each swab was plated on mannitol salt agar and incubated for 48 hours at 35°C. *S. aureus* isolates were initially screened by Staphaurex (Murex Diagnostics Inc., Norcross, GA) and identified by using standard biochemicals (6). Susceptibility testing was performed by broth microdilution (7). The patient and one of the three nurses were nasal carriers of ORSA (vancomycin MIC = 2 µg/mL). Ten days later, the nares of the carrier nurse were recultured, but no *S. aureus* carriage was detected. This health-care worker had not received topical or systemic antimicrobial therapy for *S. aureus* decolonization.

*S. aureus* strains from the patient and the health-care worker were compared by pulsed-field gel electrophoresis (PFGE) using *Sma*I- and *Eag*I-digested chromosomal DNA (Figure 1). The patient's oxacillin-resistant VISA strain was closely related by SmaI and EagI to the patient's oxacillin-susceptible strain of VISA. Although by SmaI the ORSA strain isolated from the nares of the health-care worker appeared to be related to the patient's oxacillin-resistant strain, by EagI the fragment patterns were different, and the isolates were classified as unrelated (8). Finally, the patient's oxacillin-resistant VISA strain had a similar fragment pattern by SmaI compared with the four previously reported U.S. VISA strains (2,3,9) (Figure 2).

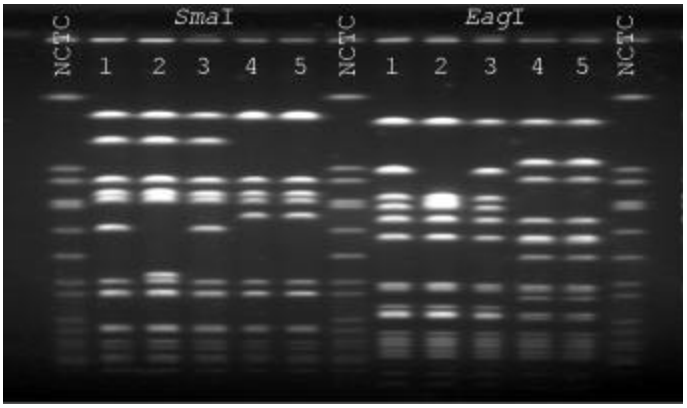


Figure 1. Pulsed-field gel electrophoresis profiles of *Sma*I- and *Eag*I-Digested DNA. NCTC, National Collection of Type Cultures 8325 control. Lane 1, patient's oxacillin-resistant vancomycin-intermediate *Staphylococcus aureus* (VISA); lane 2, patient's oxacillin-susceptible VISA; lane 3, patient's oxacillin-resistant *S. aureus* (ORSA, vancomycin MIC = 2 µg/mL) from anterior nares; lanes 4 and 5, isolates of ORSA (vancomycin MIC = 2 µg/mL) from the health-care worker's anterior nares.

## Conclusions

This case report has several unique aspects. First, each of the four previously reported U.S. patients with VISA had end-stage renal disease; this patient had normal renal function. Second, one of the patient's VISA strains did not contain the *mecA* gene and was oxacillin susceptible; previously published cases all contained the *mecA* gene. This patient's VISA strains likely emerged from the same parent strain, but one underwent a deletion to become *mecA* negative. The reasons for this are unclear and deserve further study. Third, this infection developed in the home health-care setting, not in a dialysis or acute-care setting, as did previous cases. Potential risk factors for VISA, such as prolonged exposure to vancomycin, are not limited to dialysis or acute-care settings, and clinical microbiology laboratorians and clinicians must be vigilant to recognize these strains in at-risk patients. This includes retesting *S. aureus* isolates from patients who do not respond to traditional therapy (5). Finally, this patient was successfully treated by surgery and pharmacotherapy. It is unclear if this infection would have

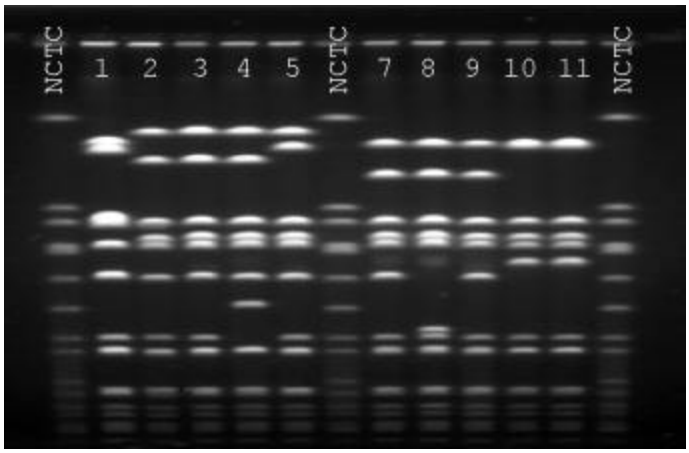


Figure 2. Pulsed-field gel electrophoresis profiles of *Sma*I digested DNA. NCTC, National Collection of Type Cultures 8325 control. Lane 1, vancomycin-intermediate *Staphylococcus aureus* (VISA) isolate from Japan, otherwise designated Mu50; lane 2, VISA isolate from Michigan (7); lane 3, VISA isolate from New Jersey (7); Lane 4, VISA isolate from New York (1); lane 5, VISA isolate from Illinois (3); lane 7, patient's oxacillin-resistant VISA; lane 8, patient's oxacillin-susceptible VISA; lane 9 patient's oxacillin-resistant *S. aureus* (ORSA, vancomycin MIC = 2 µg/mL) from anterior nares; lanes 10 and 11, isolates of ORSA (vancomycin MIC=2 µg/mL) from the health-care worker's anterior nares.

resolved with either treatment alone. The treatment of VISA infections reported to date has varied (10). The need for combination therapy is unclear, but previous reports suggest vancomycin monotherapy is inadequate (9). Until studies demonstrate efficacy of a particular regimen, clinicians must rely on the susceptibility profiles of isolates to determine which antimicrobial therapy is appropriate. Removal of prosthetic material or surgical intervention also may play an integral part in successful treatment.

Similar to previous reports, inadequate dosing of vancomycin for treatment of serious ORSA infection likely contributed to the emergence of this VISA. The limited concentration of vancomycin in bile (30% to 50% of serum levels), poor penetration of this drug into abscesses, and subtherapeutic vancomycin dosing may have favored emergence of VISA. In addition, this patient's VISA appears to have emerged from a preexisting *S. aureus* infection; however, the isolate that caused this preexisting infection was not available for study. The original ORSA isolate associated with this patient's hepatic abscess may have been acquired during her initial hospitalization or previous employment. Regardless, this VISA appears to be closely related by PFGE to the other U.S. VISA strains, suggesting certain *S. aureus* strains may have a predisposition to express the VISA phenotype.

As with previous U.S. reports, no evidence suggests that VISA spread to health-care personnel or contacts. The use of standard precautions appears to have been sufficient to prevent transmission of this strain from patient to health-care worker. All health-care agencies should use appropriate infection control practices to prevent spread of epidemiologically important organisms from patient to health-care worker and patient to patient. Having such policies in place, regardless of the setting, will protect patients and workers from unnecessary risks.

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# *Legionella*-Like and Other Amoebal Pathogens as Agents of Community-Acquired Pneumonia

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We tested serum specimens from three groups of patients with pneumonia by indirect immunofluorescence against *Legionella*-like amoebal pathogens (LLAPs) 1–7, 9, 10, 12, 13; *Parachlamydia acanthamoeba* strains BN 9 and Hall's coccus; and *Afipia felis*. We found that LLAPs play a role (albeit an infrequent one) in community-acquired pneumonia, usually as a co-pathogen but sometimes as the sole identified pathogen.

A number of bacteria that grow only within amoebae and are closely related phylogenetically to *Legionella* species, *Legionella*-like amoebal pathogens (LLAPs), have been identified and characterized (1). The role of these bacteria as human pathogens is still largely unknown. Other microorganisms, e.g., *Parachlamydia acanthamoeba* strains BN 9 (2) and Hall's coccus (3), also grow within amoebae. *Afipia felis* (once thought to be the etiologic agent of cat-scratch disease), a gram-negative rod, is difficult to grow on artificial medium but grows well in human monocytes and HeLa cells (4); this organism was recently reported to be an environmental bacterium probably associated with free-living amoebae and living in water (5). We tested serum specimens from three groups of patients with pneumonia to determine if any of these microorganisms cause disease.

## The Study

We used 511 specimens from a 1985 study of a random sample of the Nova Scotia population (6); 121 acute- and convalescent-phase serum specimens from a study (Nova Scotia, 1991-1994) of 149 ambulatory patients with community-acquired pneumonia (7); and specimens from a prospective study of community-acquired pneumonia requiring hospitalization conducted at 15 teaching hospitals in eight Canadian provinces (1996-1997).

All serum specimens from both groups of patients with pneumonia were tested for antibodies to *Mycoplasma pneumoniae*; influenza viruses A and B; parainfluenza viruses 1,2,3; adenovirus; and *Respiratory syncytial virus* (RSV) by a standard complement fixation technique in microtiter plates. Serum specimens from 60% of the patients (randomly selected from the group of patients with community-acquired pneumonia requiring hospitalization) were tested by the microimmunofluorescence test (8-10) for immunoglobulin (Ig) G and IgM antibodies to *Chlamydia pneumoniae* (AR 39 strain); *C. psittaci* (avian strain 6BC, feline pneumonitis strain FP, turkey strain TT 3, and pigeon strain CP 3); *C. pecorum* (ovine polyarthritis strain); and *C. trachomatis* (pooled antigens of serovars BED, CJHI, and FGK). Serum specimens from hospitalized patients with pneumonia were tested for antibodies to *Streptococcus pneumoniae* pneumolysin, pneumolysin immune complexes, C polysaccharide, surface protein A, *Haemophilus influenzae,* and *Branhamella catarrhalis* by Dr. M. Leinonen, National Public Health Institute, Oulu, Finland, as reported previously (11-13).

Acute- and convalescent-phase serum specimens from 150 patients also had been previously tested by enzyme-linked immunosorbent assay (ELISA) for antibodies to *L.pneumophila* serogroups 1–6 by Yu (14). A urine sample collected from each patient within 24 hours of hospitalization was tested for *L. pneumophila* serogroup 1 antigen by ELISA (15) (Binax, Inc., Portland, ME). Antibodies to *Coxiella burnetii* phase 1 and 11 antigens and to *Chlamydia pneumoniae* were determined by a microimmunofluorescence test, as described (16,17).

Antibody titers to LLAPs also were determined by the indirect fluorescent antibody technique. These included *Acanthamoeba polyphaga* strain Linc AP 1 and LLAP strains 1, 2, 4, 6, 7, 9, 10, 12; *L. lytica* (strains LLAP 3 and L2, formerly *Sarcobium lyticum*); and *Parachlamydia acanthamoeba* (strain BN 9 and Hall's coccus). *A. felis* ATCC 53690 was from the American Type Culture Collection. LLAPs were cultured in *A. polyphaga* in 150 peptone-yeast extract-glucose broth (18) at 30°C. When maximally infected, amoebae were lysed through three cycles of freeze-thawing in liquid nitrogen. This suspension was then resuspended in 30 mL of phosphate-buffered saline and centrifuged at 10,000 rpm for 10 minutes. Supernatant fluid was removed, and pellets containing respective LLAPs were resuspended in the smallest possible volume of sterile distilled water and

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were adjusted to a concentration of 2 mg/mL, as determined spectrophotometrically. Antigen prepared in this manner was frozen at –20°C until required.

Specimens with an IgM titer of ≥1:100, seroconversion from 0 to 100, a fourfold rise in antibody titer between acute- and convalescent-phase serum, and a single or stable titer of ≥400 were considered indicative of recent infection. An antibody titer of ≥50 was considered seropositive, i.e., evidence of prior but not recent infection.

The seropositivity rate to various antigens is shown in Table 1. The background rate of such infection is low. Only one healthy Nova Scotian had serologic evidence of recent infection with an LLAP, LLAP 4. None of the patients with ambulatory pneumonia had such an infection. LLAP 4 was the most common LLAP-causing pneumonia: four such infections among patients with community-acquired pneumonia required hospitalization. Two of the 58 patients from the Nova Scotia site had infection with LLAP 4, versus one of 511 healthy Nova Scotians ($p<0.029$, Fisher exact test). BN 9 caused two infections, and LLAP 1 and 12 caused one each among patients with community-acquired pneumonia requiring hospitalization.

## Case Histories

### LLAP 1 and 12 Infections

A 40-year-old floral designer was hospitalized in Edmonton, Alberta, with a 21-day history of diarrhea, myalgias, headache, chills, and shortness of breath. She had traveled to Los Angeles and Palm Springs in the previous 10 days. On admission, her oral temperature was 36.9°C, and fine bilateral interstitial infiltrates were present on a chest radiograph. She was treated with erythromycin and doxycycline, was discharged on day 7, and was readmitted 2 weeks later, at which time a transbronchial biopsy specimen yielded *Mycobacterium avium intracellulare* on culture. No evidence of HIV infection was found. The acute- and convalescent-phase antibody titers to LLAP 1 were 1:400 in the IgG fraction and 1:25 and 0 in the IgM fraction.

A 34-year-old clerical worker in a hospital radiology department was hospitalized on April 11, 1996, with pleuritic chest pain and shortness of breath of 10 days' duration. Her oral temperature was 38.7°C. The leukocyte count was $9.2 \times 10^9$ /L. A chest radiograph showed multilobar patchy opacities on the left and a 3-cm nodular opacity on the right. The patient was treated with erythromycin and cefuroxime intravenously for 36 hours, followed by oral clarithromycin. The nodule did not resolve over the next 6 weeks, and an open lung biopsy was performed. All cultures were negative. Histologic examination revealed acute and chronic inflammation. The acute-phase serum sample had an IgM antibody titer of 1:200 to LLAP 12, and the convalescent-phase titer was 1:100; the corresponding values for IgG were 0 and 1:50.

Table1.Percent seropositivity (antibody titer ≥1:50) to various antigens among three study groups[a]

| Antigen | Healthy Nova Scotians (%) N = 511 | Ambulatory pneumonia (%) N = 121 | CAP requiring hospitalization (%) N = 255 |
|---|---|---|---|
| LLAP-1 | 0.19 | 1.6 | 0.7 |
| LLAP–2 | 0 | 0 | 0.39 |
| LLAP–3 | 0.39 | 1.6 | 0.7 |
| LLAP-4 | 0.39 | 0 | 4.3 |
| LLAP–6 | 0.1 | 0 | 0.39 |
| LLAP-7 | 1.36 | 0 | 1.56 |
| LLAP–9 | 0.39 | 1.6 | 0.7 |
| LLAP-10 | 0 | 0 | 0.7 |
| LLAP-12 | 0.97 | 1.6 | 0.39 |
| Hall's coccus | 0 | 1.6 | 2.35 |
| BN 9 | 0 | 0 | 2.35 |
| *Afipia felis* | 0 | 0.82 | 0 |

[a]As defined in paper.

CAP = community-acquired pneumonia.

### BN 9 Infection

A 21-year-old university student was hospitalized with fever, abdominal pain, nausea, vomiting, diarrhea, pleuritic chest pain, and nonproductive cough. He also complained of a sore throat and shortness of breath. On examination, he looked acutely ill and had a diffuse erythematous rash. His oral temperature was 38.3°C. A chest radiograph showed diffuse opacities involving both lower lobes. He was treated with erythromycin. The next day desquamation of the lips and the skin of the digits was noted, and a diagnosis of adult Kawasaki disease was entertained. Treatment with aspirin and gamma globulin was instituted, and the patient made an uneventful recovery. There was no evidence of cardiac involvement as indicated by normal serial electrocardiograms and a normal echocardiogram. The BN 9 antibody titer was 1:50 and 1:6,400 in the acute- and convalescent-phase serum specimens. There was a stable antibody titer to Hall's coccus of 1:400 in both. Blood and urine cultures, as well as other microbiologic tests were negative.

A 68-year-old man was hospitalized on October 15, 1996, with nausea, vomiting, diarrhea, a nonproductive cough, shortness of breath, chills, and pleuritic chest pain. The year before, he had received a cadaveric renal transplant and was maintained on corticosteroid and cyclosporin therapy. His oral temperature was 39.2°C, and consolidation was found on examination of the right lung. A chest radiograph showed a single lobar opacity on the right. The leukocyte count was $17 \times 10^9$/L. *S. pneumoniae* was isolated from the sputum. The patient was treated with cefuroxime intravenously for 4 days and was discharged on oral cefaclor. He made an uneventful recovery. The acute- and convalescent-phase titers to BN 9 were stable at 1:400.

### LLAP 4 Infection

Four patients met our definition for infection with LLAP 4 (Table 2). Appearance of pneumonia was similar in all four chest radiographs. Patient ML 13 had diffuse interstitial infiltrates, but this patient, who had had a bone marrow transplant, also had RSV infection. All patients with LLAP 4 recovered from pneumonia.

## Conclusions

In August 1986, Rowbotham (19) isolated LLAP 1 from the sputum of an 82-year-old woman with persistent

Table 2. Summary of selected characteristics of four patients infected with *Legionella*-like amoebal pathogen (LLAP)–4

| No. | Age | Sex | Temp | Symptoms | LOS | WBC | Antibiotics | Comments | Antibody titer | Co-pathogens |
|---|---|---|---|---|---|---|---|---|---|---|
| TM37 | 93 | M | 38.3 | nausea, myalgia, shortness of breath, nonproductive cough, chest pain | 8 | 18.2 | erythromycin; cefuroxime | admitted from a nursing home, sustained a non q wave myocardial infarction | IgG 1:400 and 1:400 | None |
| TM11 | 87 | F | 39.0 | nonproductive cough, chills | 11 | 8.7 | erythromycin; cefuroxime | | IgG 0 and 1:100 | None |
| ML13 | 54 | M | 38.4 | abdominal pain, nonproductive cough, myalgia, chest pain | 17 | 2.2 | erythromycin; ceftazidime, ribavirin | bone marrow transplant; required intensive care unit treatment | IgM 25 and 0; IgG 200 and 1:400 | RSV |
| LN 9 | 63 | M | 36.8 | shortness of breath, chills | 10 | 20.8 | erythromycin, cefuroxime | | IgM 1:200 and 1:200 | *Strepto-coccus pneumoniae* |

M = male; F = female; LOS = length of stay in days; wbc = leukocyte count; antibody titer = first value is from acute-phase sample and second from convalescent-phase sample.

pneumonia, by cocultivation with *A. polyphaga*. Seroconversion was demonstrated to LLAP 3. He screened >5,000 serum specimens submitted for *Legionella* antibody testing and found that 10 patients met the criteria for infection with LLAP 3 (19).

The only other study similar to ours is a study by Benson et al. (20), who examined 500 patients with community-acquired pneumonia and determined antibody titers to LLAP 1,2,3,4,6,7,9, and Hall's coccus; 94 (18.9%) had a fourfold rise in antibody titer of $\geq$128 to any LLAP; 36 (7.2%) had a titer rise to $\geq$1,024. In contrast, 1.4% of our 255 hospitalized patients with community-acquired pneumonia had evidence of recent infection with a LLAP or Hall's coccus. As in our series, LLAP 4 was the most common cause of infection in the Benson study, which also found that in 10 (10.6%) of 94 patients with LLAP or Hall's coccus infection a copathogen had been implicated as cause of the pneumonia. Likewise, almost all our patients with LLAP infection were infected with another pathogen.

One of the most interesting findings in our study was a fourfold rise in antibody titer to BN 19 in a patient with presumed adult-onset Kawasaki syndrome, an acute vasculitis of unknown cause found predominantly in infants and young children. The diagnostic criteria include fever of >5 days plus four of the following five features: bilateral conjunctivitis without exudate; polymorphous eruption; cervical lymph node >1.5 cm in diameter; changes in the extremities, including edema of the hands or feet, palm or sole erythema, and periungual desquamation during convalescence; and changes in the oropharynx, including fissured red lips, strawberry tongue, and diffuse erythema of the oropharyngeal mucosa (21). Our patient met this definition. An association between an antecedent respiratory infection and Kawasaki syndrome has been described (21,22), as has exposure to freshly cleaned carpets (23,24). It is possible that the gamma globulin administered to our patient contained antibody to BN 19. However, there was no seroconversion or high titer of antibody to any of the other antigens included in our test panel. A possible association between infection with BN 19 and Kawasaki syndrome is easily tested.

Strengths of this study are its size and the comprehensiveness of the diagnostic work-up. Its limitations include the following: the three populations were enrolled in different periods; we tested only a subset of the patients hospitalized with community-acquired pneumonia and these patients were from multiple centers across Canada; our comparison groups (healthy persons and patients with ambulatory pneumonia) were Nova Scotians. However, the inferences that we are making are limited to the rate of infection in these three separate groups and are not intended to indicate differences temporally or geographically.

Our data suggest that LLAPs play a role, albeit an infrequent one, in community-acquired pneumonia. Usually they are a copathogen, but in some cases they are the sole pathogen. The possible association between BN 9 and Kawasaki disease requires further study.

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# Absence of High-Level Vancomycin Resistance in Enterococci Isolated from Meat-Processing Facilities

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Enterococci isolated from packaging areas of meat-processing facilities that produce ready-to-eat meat products were examined for high-level vancomycin resistance. A total of 406 enterococci isolates from the plants' packaging areas were examined for vancomycin resistance. High-level vancomycin resistance was not demonstrated in any enterococci isolated from 12 meat-processing plants.

Since 1989, vancomycin-resistant enterococci (VRE) have emerged as important nosocomial pathogens. In the United States, the prevalence of VRE in hospitals has increased from 0.3% in 1989 to 11%-13% by 1996 in patients other than those in intensive care units (1). Currently in the United States, more than half of all clinical isolates of *Enterococcus faecium* are not treatable with vancomycin (2). Another concern is that, as enterococci are found in the gastrointestinal tract of humans and animals, they may serve as a reservoir of glycopeptide resistance genes that may be transferred to other organisms such as methicillin-resistant *Staphylococcus aureus* and *S. epidermidis*.

The source of VRE is not known, although two potential reservoirs for these organisms are hospitals and food animals fed growth promoters, such as avoparcin (3,4). Padiglione et al. (5) found that human fecal colonization with VRE is uncommon in Australia, despite the relatively high level of consumption of avoparcin by industry (10,000 kg/year). In Europe, there have been numerous reports of VRE isolated from food animals and food products (4,6,7). In the United States, Knudtson and Hartman (8) studied the prevalence of antimicrobial resistance in enterococci from water, pork, and clinical isolates. In their studies, no VRE was detected from pork carcasses or fresh or spoiled pork products. Also in the United States, Coque et al. (9) and Thal et al. (10) studied enterococci from animal sources and did not recover VRE from the samples examined.

Avoparcin was licensed in Europe in 1975 and subsequently banned throughout the European Union in 1997 (6). In Australia, the per capita consumption of avoparcin by Australian agriculture is one of the highest in the world (5), while in the United States avoparcin has never been licensed for animal feed. The incidence of VRE infections in European countries is relatively low compared with the high, increasing rate in the United States, indicating that clinical use of vancomycin may be the reason for differences observed between the two populations.

The food chain has been proposed as a suspected source for dissemination of VRE to the human population (6). Should this be the case, one possible source is plants that produce ready-to-eat foods. Enterococci can be found in the environment of food-processing facilities, including those producing ready-to-eat meat and poultry products, but the incidence of vancomycin resistance among enterococci from these facilities is not known. We investigated the presence of VRE in 12 meat-processing plants in 8 U.S. states.

## The Study

A total of 446 swabs of floors and surfaces (in areas with and without food contact) were obtained from 12 meat-processing plants in 8 U.S. states during 1999. All processing facilities produced ready-to-eat meat products. The number of swabs collected at each plant ranged from 8 to 73. Swabs were taken by using either a sterile gauze pad or a sterile sponge moistened with Butterfield's phosphate diluent. The area covered by the swab depended on the sampling site. For sampling floors, an area of approximately 1 square meter was covered. All swabs were sent to the testing laboratory within 24 hours of collection. Swab samples were enriched in University of Vermont medium (UVM) and incubated at 30°C for 24 hours. Following enrichment, 0.1 mL of UVM culture was transferred to Fraser broth for selective enrichment and incubated at 35°C for 24 hours. All tubes that had visible growth or a darkened appearance due to esculin hydrolysis were streaked onto Bile Esculin Azide agar (BEAA) and incubated at 35°C for 48 hours. A single typical colony was selected from each sample and identified to genus level on the basis of esculin hydrolysis, bile tolerance, and colony morphology on BEAA. Enterococci were screened for vancomycin resistance by the method of Klein et al. (3) with some modifications. Enterococci isolates were subcultured onto Mueller-Hinton agar supplemented with 32 µg/mL vancomycin (MHV) to screen for high-level vancomycin resistance. Sensitivity of the isolate was determined by the confluence of bacterial growth on MHV plates after 48 hours of incubation at 35° C. No growth indicated sensitivity to vancomycin.

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## Conclusions

The increasing prevalence of VRE in the United States is generally attributed to the hospital use of antibiotics (2,11). The meat-processing facilities examined in this study all produce ready-to-eat products made from beef, pork, or poultry. A total of 406 enterococci isolates from the plants' packaging areas were examined for high-level vancomycin resistance: 202 were isolated from food-contact areas and 134 from other areas and floors. No high-level vancomycin resistance was demonstrated in any enterococci isolated from the 12 meat-processing plant environments. These data suggest that enterococci with VanA resistance phenotype are uncommon in U.S. meat-processing facilities producing ready-to-eat meat products.

Dr. Bodnaruk was formerly employed by ConAgra Refrigerated Prepared Foods, Downers Grove, Illinois, as director of microbiology. He is currently microbiology section leader with Food Science Australia in Sydney. His major interests are innovative food-processing technologies and control of emerging food safety hazards.

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# Community-Acquired *Acinetobacter radioresistens* Bacteremia in an HIV-Positive Patient

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We describe the first case of community-acquired bacteremia caused by *Acinetobacter radioresistens*; the patient was a 32-year-old HIV-positive neutropenic woman. Ambiguous Gram staining and poor biochemical reactivity of blood culture isolates misguided early diagnosis and therapy. Bacterial identification was based on 16S rDNA sequence analysis. *A. radioresistens* can be considered as a cause of opportunistic infection in immunodeficient patients.

Members of the genus *Acinetobacter* are described as gram-negative, strictly aerobic diplococcoid rods that are oxidase negative and catalase positive (1). The genus includes at least 19 genomic species, defined on the basis of DNA relatedness criteria (2), which are ubiquitous in nature and have become increasingly responsible for a range of systemic infections in critically ill and immunocompromised patients (3). Genospecies 1 (*A. calcoaceticus*), 2 (*A. baumannii*), 3, and 13TU, classified as the *Acb complex*, are prevalent in nosocomial pneumonia and bacteremia but rarely colonize healthy persons (3,4). Genospecies 8/9 (*A. lwoffii*), 15BJ, and 12 (*A. radioresistens*) constitute part of the normal skin microflora but are seldom associated with human infections (5).

*Acinetobacter* spp. are responsible for 1%-2% of nosocomial bloodstream infections (4,6), in which *A. baumannii* represents the most commonly isolated species (3,7). Few *Acinetobacter* bacteremias are community acquired (8). The respiratory system and vascular devices are the main portals for entry of *Acinetobacter* into the bloodstream of critically ill persons (9). Secondary bloodstream invasion, resulting from dissemination of the bacterium from covert colonization sites, can also be considered when evidence of primary infection is missing (10). The outcome of *Acinetobacter* bacteremia is usually benign, with the prognosis depending on the severity of underlying disease(s) and the efficacy of antibiotic therapy (7-10).

In most clinical microbiology laboratories, identification of *Acinetobacter* cannot routinely be achieved at the genospecies level because commercial identification systems are substantially deficient and poorly discriminatory in distinguishing these organisms. This implies that local data on the prevalence of individual species in human infections should be interpreted cautiously unless supported by DNA-based taxonomy. Here we report a case of community-acquired *A. radioresistens* bacteremia in an HIV-positive patient, in which the causative agent was identified by means of 16S ribosomal DNA (rDNA) sequencing.

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## The Study

In January 2000, a 32-year-old HIV-positive woman was admitted to the National Institute for Infectious Diseases "L. Spallanzani," Rome, with a 10-day history of fever, productive cough, headache, rhinitis, and muscular pain. She tested HIV positive in 1993, citing heterosexual risk factors. In December 1999, she had HIV viremia of <80 copies/mL and a $CD4^+$ cell count of 309/$mm^3$. She had never taken antiretroviral therapy and had not been on antibiotic treatment in the previous 6 months.

The patient's recent history included chronic left suppurative otitis media with ear drainage and recurrent attacks of headache. One week before admission she had undergone computerized tomography scans of the brain, with contrast infusion; the scans were normal.

On admission the patient had fever (37.8°C), pallor, headache, left ear pain, and hearing loss. Lung examination revealed sparse crackles, but the chest radiograph was normal. Laboratory values were significant for leukopenia, with a leukocyte count of $2.5x10^3/mm^3$ (normal range 4.3-$10.8x10^3/mm^3$), and neutropenia ($1.0x10^3/mm^3$; normal range 1.4-$7.5x10^3/mm^3$). C-reactive protein (CRP) was 2.1 mg/L (normal values <6 mg/L), erythrocyte sedimentation rate (ESR) 66 mm in the first hour (normal values <15 mm per hour), and platelet count $118x10^3/mm^3$ (normal range 140-440/$mm^3$). The urine was normal, as were electrolytes, glucose, hemoglobin, and creatinine. X-ray examination of the sinuses revealed thickening of the right mucous membranes. Otoscopy revealed chronic left middle ear disease with acute inflammation. Two blood cultures, taken 3 and 6 hours after admission, were negative.

After 2 days in hospital, the patient returned home against the advice of the physicians. At home she was feverish and had continuous headache and reoccurence of left ear pain. One week later she was readmitted with fever (39.1°C), leukopenia ($2.5x10^3/mm^3$), neutropenia ($0.7x10^3/mm^3$),

higher CRP levels (3.2 mg/L), and accelerated ESR (70 mm per hour). Physical examination showed no substantial changes compared with one week earlier. Because of the patient's symptoms and the results of otoscopy, a diagnosis of chronic middle ear disease was made, and she was treated empirically with intravenous ceftriaxone (2 g once a day) and gentamicin (80 mg three times a day).

Three days later, five of six blood cultures taken at 3-hour intervals during the first day of her second admission yielded visible bacterial growth. Gram staining of blood-free supernatants from all positive cultures was interpreted as showing a homogeneous smear of gram-positive diplococci and was used as the inoculum of Sceptor gram-positive Breakpoint/ID panels (Becton Dickinson, Franklin Lakes, NJ). Individual isolates from all five positive blood cultures showed an identical antibiotic-susceptibility pattern; they were resistant to penicillin, oxacillin, amoxicillin/clavulanate, clarithromycin, clindamycin, chloramphenicol, erythromycin, vancomycin, and teicoplanin, but sensitive to aminoglycosides, carbapenems, cephalosporins, ciprofloxacin, cotrimoxazole, and tetracycline. Bacterial identification could not be achieved because of lack of biochemical reactivity of the strain. Previous antibiotic therapy was discontinued, and intravenous ciprofloxacin (400 mg twice a day) was begun for 2 weeks.

All blood culture isolates grew vigorously at 37°C on both chocolate agar and Columbia agar base supplemented with 5% (vol/vol) sheep blood, giving similarly smooth, opaque, nonhemolytic colonies. Tiny colonies appeared on eosin-methylene blue (EMB)-lactose agar after 36-48 hours' incubation. The presence of gram-positive coccobacillary forms, mostly organized in pairs, was confirmed for primary isolates. Growth was not detected on either mannitol salt agar or D-coccosel agar or, under anaerobic conditions, on Columbia agar base. Catalase and oxidase reactions were positive and negative, respectively. Infection by *Alloiococcus otitidis* was initially suspected, but specific biochemical tests and antibiotic susceptibility data argued against this hypothesis (data not shown).

Bacterial identification was achieved by means of 16S ribosomal DNA (rDNA) sequence analysis. Genomic DNA was extracted from each of the five isolates with a commercial kit (Quiagen genomic-tip, Qiagen Inc., Valencia, CA), and polymerase chain reaction (PCR) amplification was performed with universal primers annealing at the extreme 5' and 3' ends of the eubacterial 16S rDNA (encompassing nucleotides 9-27 and 1492-1512 relative to the *Escherichia coli* 16S rDNA sequence, International Union for Biochemistry [IUB] nomenclature) (2). The 16S rDNA amplicon was purified with the QUAquick PCR purification kit (Qiagen) and partially sequenced on one strand from the 5'-end using an ABI PRISM 377 (PE Applied Biosystems, Foster City, CA) automated sequencer and dye-labeled dideoxy chain-terminator chemistry (Dye Terminator Cycle sequencing Ready Reaction Kit, Applied Biosystems Inc.). Identical partial sequences were obtained for all the five amplicons analyzed, corresponding approximately to nucleotides 60-500 of the *E.coli* 16S rDNA gene sequence. Comparative BLAST software (version 2.0, National Center for Biotechnology Institute, http://www.ncbi.nlm.nih.gov/BLAST/) analysis with entries available at the EMBL, GenBank, and Ribosomal Data Project (http://www.cme.msu.edu/RDP/) databases retrieved an optimum alignment (99.4% identity) with the 16S rDNA of the *A. radioresistens* type strain M17694 (GenBank sequence accession number Z93445; ref. 2), and an excellent match with the published *A. radioresistens* 16S rDNA signature regions (Table). The sequence within the hypervariable helix 6 showed a G/A mismatch at position 75, compared with the published *A. radioresistens* sequence (2). However, the same single-base difference was found in the corresponding 16S rDNA signature of the partial sequence recently deposited under the accession number AJ247210, corresponding to *A. radioresistens* LMG 10614 (Harmsen D, Singer C, unpub. data).

Biochemical identification was repeated with the Sceptor gram-negative Breakpoint/ID and API 20NE panels. Both systems misidentified the organism as *A. lwoffi* (Sceptor and API codes were 0000000 and 0000032, respectively), although the combined results of both biochemical and assimilation tests were compatible with the identification as *A. radioresistens*.

Ten days after beginning the course of ciprofloxacin, the patient improved symptomatically, her temperature subsided, and serologic markers of inflammation declined (CRP and ESR values were 0.9 mg/L and 25 mm, respectively). She was discharged from hospital 4 days later, and she had no recrudescence of otitis or bacteremia in a 3-month follow-up period.

Possible sources of contamination were retrospectively investigated and ruled out. Infection control procedures in the unit were reviewed, and sterility control of 24 randomly sampled blood culture bottles from the same batch gave negative results. Moreover, no other strains similar to *A.radioresistens* were isolated in our institute from November 1999 to March 2000.

## Conclusions

To our knowledge, this is the first description of *A.radioresistens* causing community-acquired bacteremia. We speculate that systemic disease developed in our patient as a result of local infection; the combination of neutropenia and her impaired immunologic condition due to HIV infection made her susceptible to the infection.

Paranasal sinuses and the middle ear are potential reservoirs from which bacteria, including *Acinetobacter* spp., can enter the bloodstream; otitis media and sinusitis often precede bacteremia in predisposed patients (11 and references therein). Thus, we speculate that the left middle ear was the most likely portal for the entry of *A. radioresistens* into the bloodstream of the patient, although other sites cannot be ruled out. The history of recurrent episodes of ear drainage and the rapid remission of signs and symptoms following targeted antimicrobial therapy point to the middle ear infection as a plausible source for the systemic spread of *A. radioresistens*. Unfortunately, no clinical specimen was obtained for culture from the middle ear of the patient to confirm the diagnosis.

A Gram stain of bacteria from positive blood cultures is considered to be an important guide for the etiologic diagnosis and initial antibiotic choice. However, *Acinetobacter* spp. are known for being extremely resistant to decolorization (1), and diagnostic errors due to misinterpretation of well-prepared Gram stains have been reported (12). In our case, the gram-positive appearance of primary cultures of

Table. Sequence motifs of the variable regions for *Acinetobacter* 16S rRNAs, encompassing positions 70-101 (helix 6) and 453-477 (helix 18)[a]

| DNA group | Helix 6 variable region | Helix 18 variable region |
|---|---|---|
| 1 | GGAAGGUUGCUUCGGUAACUGACCUA | GCUCUCUUAGUUAAUACCUAAGAUG |
| 2 | GGGAAGGUAGCUUGCUACCGGACCUA | CCUACUUUAGUUAAUACCUAGAGAU |
| 3 | AGAGAGGUAGCUUGCUACUGAUCUUA | GCUACUUUAGUUAAUACCUAGAGAU |
| 4 | GGAAGGGUACCUUGCUACCUAACCUA | GCUACUCUAGUUAAUACCUAGAGAU |
| 5 | AGAUGAGGUGCUUGCACCUUAUCUUA | GCUACUGAGACUAAUACUCUUGGAU |
| 6 | GGUGAUGUAGCUUGCUACAUUACCUA | GCUACCUAGACUAAUACUCUAGGAU |
| 7 | GGAGAGGUAGCUUGCUACCUAACCUA | GCUACUUGGAUUAAUACUCUAGGAU |
| 8 | GGAGAGGUAGCUUGCUACAUAACCUA | GCUACCGAGAUUAAUACUCUUGGAU |
| 9 | GGAAGNGUAGCUUGCUACAUAACCUA | GCUACCGAGAUUAAUACUCUUGGAU |
| 10 | GGGAGAUUGCUUCGCUAAUUGACCUA | GCUCUUUUGGUUAAUACCCAAGAUG |
| 11 | GGGAGAUUGCUUCGGUAACUGACCUA | CCUCUCUUGGUUAAUACCCAAGAUG |
| 12 | AUGAA**G**GUAGCUUGCUACUGGAUUCA | GCUACCUAGAUUAAUACUUUAGGAU |
| AJ247210 | AUGAA**A**GUAGCUUGCUACUGGAUUCA | GCUACCUAGAUUAAUACUUUAGGAU |
| AR | AUGAA**A**GUAGCUUGCUACUGGAUUCA | GCUACCUAGAUUAAUACUUUAGGAU |
| TU13 | GGGAAGGUAGCUUGCUACUGGACCUA | GCUACUCUAGUUAAUACCUAGGGAU |
| TU14 | GGAAGGGUAGCUUGCUACCUAACCUA | CCUACCUAGAUUAAUACUCUAGGAU |
| TU15 | GGAUAGGUUGCUUGCACUUGAUGCUA | GCUUACCUGGUUAAUACCUGGGAUA |
| CTTU13 | GGAGAGGUAGCUUGCUACUGAUCUUA | GCUACUUUAGUUAAUACCUAGAGAU |
| 1-3 | GNUGAUGGUGCUUGCACUAUCACUUA | GCUACUUUAGUUAAUACCUAGAGAU |
| BJ14 | GGAAGGUUGCUUCGGUAUCUGACCUA | GCUCUCUUAGUUAAUACCUAAGAUG |
| BJ15 | AGUUAUGGUGCUUGCACUAUGACUUA | GCUCUCUUAGUUAAUACCUAAGAUG |
| BJ16 | AGUGAUGGUGCUUGCACUAUCACUUA | GCUACUAGUACUAAUACUACUGGAU |
| BJ17 | AGUGAUGGUGCUUGCACUAUCACUUA | GCUCUCCUAGUUAAUACCUAGGAUG |

[a]Representative strains for each DNA group (1 to 12, TU13 to TU15, CTTU13, 1-3, BJ14 to BJ17) are those listed in ref. 2. The designations AJ247210 and AR refer to *A. radioresistens* LMG 10614 (genospecies 12) and to our isolate, respectively. Nucleotides in bold highlight the differences between members of genospecies 12.

*A.radioresistens* delayed bacterial identification, and it was not until the organism was later observed growing on EMB agar that an incorrect diagnosis was suspected. Cases of *A. radioresistens* infection may be underestimated because this species escapes routine detection by most commercially available microbiologic tests (*A. radioresistens* is not included in the Sceptor version 3.10 database and in the API 20NE analytic catalog, 6th edition, 1998). Bacterial identification based on 16S rDNA sequence analysis can be performed directly on monomicrobic blood cultures and can be completed within 36 hours at relatively low cost. This case highlights the power of this technique for the rapid and correct identification of *A. radioresistens*.

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# A Cultured Strain of "*Helicobacter heilmannii*," a Human Gastric Pathogen, Identified as *H. bizzozeronii*: Evidence for Zoonotic Potential of *Helicobacter*

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We compared the characteristics of a cultured human "*Helicobacter heilmannii*" isolate with those of other helicobacters found in animals. Phenotypic, protein profile, 16S rDNA sequence, and DNA-DNA hybridization analyses identified the human strain as *H. bizzozeronii*, a species frequently found in dogs. Thus, *H. bizzozeronii* may have zoonotic potential.

The importance of *Helicobacter pylori* in human gastric disease is well recognized (1). After its initial isolation, a distinctive, tightly coiled organism was also observed in some human gastritis cases (2). Although these organisms could not be isolated in vitro (3,4), they were designated as "*Gastrospirillum hominis*" on the basis of morphology and ecologic niche (4), and later as "*H. heilmannii*" on the basis of 16S rRNA gene sequence analysis. Differences in 16S rRNA gene sequences have allowed two types to be defined for "*H.heilmannii*," which accounts for 0.2% to 4% of human gastritis cases (5). Evidence suggests that infections with this organism may be more frequently associated with mucosa-associated lymphoid tissue lymphomas (6).

Animals, especially cats, dogs, and pigs, are frequently infected with large spiral organisms (often referred to collectively as gastrospirilla) that closely resemble "*H. heilmannii*" strains in humans (3,7-10). As a consequence, the possibility of zoonotic infection has been considered (3,10,11). Three distinct species resembling "*H. heilmannii*" have been isolated from cats or dogs, namely *H. felis, H. bizzozeronii,* and *H.salomonis* (10). The provisional name "*Candidatus* H. suis" has been proposed for the pig organism (8). However, critical comparison of strains from each of these sources has not been possible because few workers have obtained cultures of human strains. To our knowledge, only one "*H. heilmannii*" strain has been cultured from human tissue (6).

We present the results of a polyphasic identification analysis that confirms this human "*H. heilmannii*" isolate as *H. bizzozeronii*, and we discuss the public health implications of this finding.

## The Study

The human "*H. heilmannii*" isolate, Rigshospitalet 53 (R-53), and other *Helicobacter* strains for comparison were cultured by using conditions described earlier for phenotypic (12), whole-cell protein (13), 16S rRNA sequence (12), and DNA-DNA hybridization (10) analyses. A total of 66 phenotypic tests were determined for R-53, and results were compared both empirically and by numerical analysis with similar data for 116 other strains of *Helicobacter*, as described (12). Primers and methods used for polymerase chain reaction (PCR) amplification and direct sequencing of the full 16S rRNA gene were as described previously, as were the methods used for sequence alignment and phylogenetic analysis (14). The whole genomic DNA was isolated and dot-blot DNA-DNA hybridizations were performed (10), with DNA derived from R-53 used as a probe to characterize the type strains of *H. felis*, *H. bizzozeronii*, and *H. salomonis*. Whole-cell protein samples were prepared, separated by polyacrylamide gel electrophoresis (running length 9 cm), digitized, and subjected to comparative numerical analysis(13).

The phenotypic characteristics of R-53 were consistent with those documented for *H. felis*, *H. bizzozeronii*, and *H.salomonis* (10), with two principal differences: the cultures formed distinct colonies, and the growth exhibited was profuse with a greasy surface film. This morphology is typical of *H. pylori* (1) but differs from the sparse growth lacking defined colony formation observed in cultures of the other *Helicobacter* species mentioned (10). Furthermore, R-53 grew weakly on 1.0% and 1.5% ox-bile media. This trait is rare among gastric helicobacters but more common in species colonizing the lower intestine (15). Nonetheless, numerical comparison of phenotypic characters demonstrated that R-53 was most similar (90.9%) to a reference strain (Wiberg) of *H. bizzozeronii*. These two strains formed part of a larger cluster delineated at the 87% similarity level containing all 12 *H. bizzozeronii* and six *H. salomonis* isolates studied, as well as 12 of 15 *H. felis* and an *H. muridarum* strain (a mouse isolate). This cluster was clearly differentiated from all other *Helicobacter* species, including 14 type and reference strains of *H. pylori* obtained from humans, monkeys, and a pig (data not shown).

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A phylogenetic tree based on comparisons of 16S rRNA gene sequences confirmed previous observations (6) that R-53, *H. felis*, *H. bizzozeronii*, *H. salomonis*, and "*H. heilmannii*" type 2 were highly related and formed a distinct cluster within the rRNA homology group III (i.e., the *Helicobacter* phylogenetic branch) of rRNA superfamily VI (data not shown). Although the infra- and intraspecific sequence variation observed among these species makes unequivocal differentiation between them impossible, these species are readily distinguished from other helicobacters, including the "*H.heilmannii*" type 1 taxon. The closest phylogenetic neighbor of the latter is "*Candidatus* H. suis" (8), which may be found in up to 87% of pigs (7). The complex relationships observed within the "*H. heilmannii*" type 2-containing clade exemplify cases in which 16S rDNA sequence comparisons cannot be used as an accurate species identification method, as also seen with the related taxa *H. cinaedi* (16) and *C. hyointestinalis* (14).

Numerical comparison of protein profiles of R-53 with those of all extant *Helicobacter* species placed this strain in a cluster with type and reference strains of *H. bizzozeronii* (Figure 1). This clustering confirms the earlier finding that species identification of cultured *Helicobacter* strains is efficiently performed with highly standardized whole-cell protein analysis, and the results are in excellent agreement with those of DNA-DNA hybridization (10,13,16). Dot-blot DNA-DNA hybridization demonstrated that the R-53 probe did not hybridize intensively with genomic DNA of either *H. felis* or *H. salomonis*, but hybridized strongly with DNA from all isolates of *H. bizzozeronii*, including homologous assays (Figure 2). Therefore, compilation of the phenotypic and genotypic data determined for R-53 clearly identify it as a strain of *H. bizzozeronii*, the most common *Helicobacter* species (approximately 44% of isolates) found in healthy and diseased pet dogs (10).

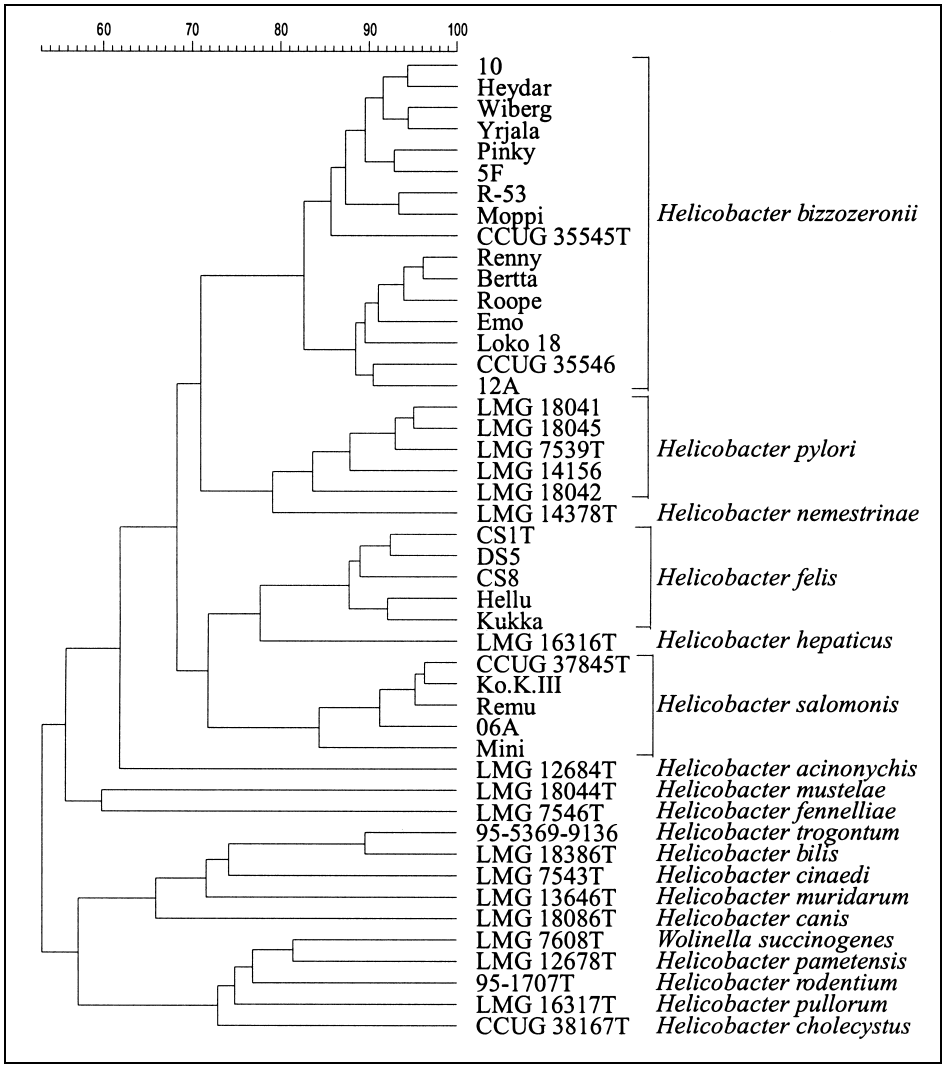


Figure 1. Dendrogram expressing similarity in whole-cell protein patterns of strain R-53, and type and reference strains of all *Helicobacter* and *Wolinella* species.

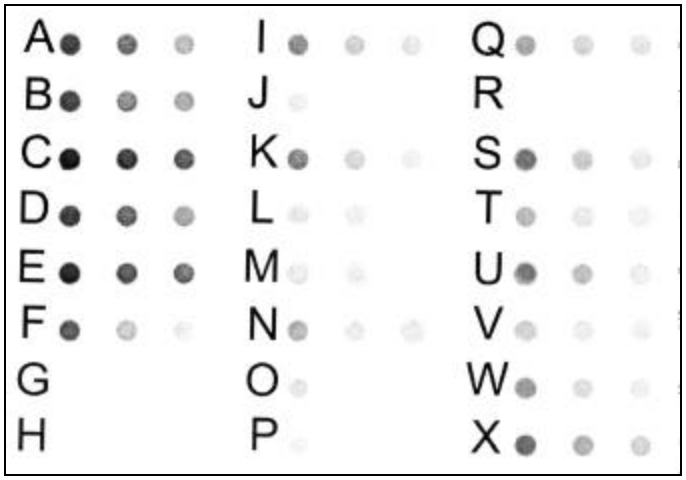


Figure 2. Dot-blot DNA-DNA hybridization assay with R-53 as a probe. A-F *Helicobacter bizzozeronii* strains: A. R-53, B. CCUG 35545$^T$, C. CCUG 35546, D. 12A, E. 10, F. Poju; G-M *H. felis* strains: G. CS1$^T$, H. DS3, I. CCUG 37471, J. 15/390, K. Dog 1, L. Dog 3, M. CS2; N-P *H. pylori* strains: N. Tx30, O. 26695, P. CCUG 17874$^T$; Q. *H. mustelae* CCUG 25715$^T$; R. *H. acinonychis* CCUG 29263$^T$; S-X *H. salomonis* strains: S. 06A, T. CCUG 37845$^T$, U. Vilho, V. Mini, W. Remu, X. Ko.K. III.

## Conclusions

Although further studies are needed to clarify the taxonomic relationships between "*H. heilmannii*" type 1, "*Candidatus* H. suis," and members of the *H. felis-bizzozeronii-salomonis* species complex, available data strongly indicate that human infections with gastrospirilla may be acquired from more than one source, including pigs, dogs, and cats. Several epidemiologic studies have shown an increased incidence of "*H. heilmannii*" in humans associated with animal contacts and these bacteria (11,17). PCR-restriction fragment-length polymorphism (RFLP) analysis of the *Helicobacter ureB* gene performed directly on stomach biopsy

tissue showed the presence of multiple types of gastrospirilla in a single patient; the same PCR-RFLP type was also identified in the patient's cat (3). In conclusion, this study clearly demonstrates the zoonotic potential of large, spiral gastric *Helicobacter* spp. The only human "*H. heilmannii*" isolate to date was identified in a polyphasic taxonomic analysis as *H.bizzozeronii*. More studies with a number of isolates are needed to evaluate the full zoonotic potential of animal *Helicobacter* spp. Given the difficulties in culturing these organisms in vitro, available data strongly indicate the need for species-specific PCR assays for detecting the various *Helicobacter* spp. that inhabit the gastric mucosa of humans and animals.

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Dr. Jalava, whose research interests focus on the taxonomy of animal helicobacters, works in the National Public Health Institute of Finland. This study was carried out in the Department of Food and Environmental Hygiene of the University of Helsinki.

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# Nontuberculous Mycobacterial Disease Following Hot Tub Exposure

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Nontuberculous mycobacteria (NTM) have been recognized as an important cause of disease in immunocompromised hosts. Pulmonary disease caused by NTM is increasingly recognized in previously healthy persons. Investigation of pulmonary disease affecting a family of five identified an indoor hot tub as the source of NTM-related disease.

Nontuberculous mycobacteria (NTM) are an important cause of disease in the United States, with the number of NTM isolates exceeding those of *Mycobacterium tuberculosis* (1). Pulmonary disease, the most commonly reported localized manifestation of NTM, is often associated with the *M.avium* complex (MAC) (2). Other NTM species, such as *M. kansasii, M. fortuitum, M. xenopi,* and *M. abscessus,* have also been associated with pulmonary disease (2,3). Although NTM-associated pulmonary disease has been described primarily among immunocompromised persons (4,5), it is being recognized with increasing frequency among those without predisposing conditions (2,6,7).

Unlike MTB, NTM are not known to be transmitted person to person. Most NTM have been isolated from water or soil (8-14). Species such as MAC are thermophilic (12), resistant to chemical germicides (14), and readily aerosolized (13). For several NTM species, environmental sources have been linked epidemiologically to cases of disease (15-20). In 1991, Burns investigated an outbreak of respiratory tract colonization in which epidemiologic and pulsed-field gel electrophoresis (PFGE) findings implicated a contaminated showerhead as the source of *M. fortuitum* (21). Subsequently, von Reyn used PFGE to link MAC infection in five AIDS patients to hot water sources in two hospitals (22).

Recently, Embil et al. (23) described five persons who became ill with pulmonary disease following exposure to hot tubs. MAC was isolated from all five patients and the two tubs. When MAC isolates were examined by multilocus enzyme electrophoresis (MEE), however, the hot tub and patient isolates had different MEE patterns. Kahana et al. (24) reported one patient diagnosed with MAC disease associated with a hot tub. In this case, the organisms isolated from the patient and the tub were identical by MEE.

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## The Study

In October 1998, the Boulder County Health Department and the Tuberculosis Program, Colorado Department of Public Health and Environment, began an investigation into an apparent cluster of tuberculosis cases among a previously healthy family of five.

Case 1: This patient, a 46-year-old woman, was in excellent health until early June, when she noted the onset of shortness of breath and a dry cough. A chest radiograph at that time was consistent with early right lower lobe bronchial pneumonia. The patient was treated over the subsequent weeks with a series of antibiotics, including amoxacillin and azithromycin, without improvement. During early July, she had fever as high as 104°F approximately every 4 days, accompanied by night sweats and preceded by chills. A chest radiograph showed increased markings in the right lower lobe medially with associated peribronchial thickening and some faint air bronchograms consistent with an early bronchial pneumonia. She was begun on a course of tetracycline and prednisone (50 mg four times a day for 3 days, decreased to 20 mg four times a day for 11 days). During this time the patient traveled to Disneyland and while away felt much improved. Her symptoms recurred with the cessation of steroids and return home, however, and by the end of August, her shortness of breath, night sweats, and malaise had worsened, and she had an 18-pound weight loss.

Before she became ill, the patient had exercised regularly. In late August, her shortness of breath worsened so that she was unable to walk across a room, and she visited the hospital. A chest radiograph showed increased interstitial markings in both lungs. A computed tomographic (CT) scan of the chest showed a diffuse increase in pulmonary interstitial markings, with a ground-glass background. On September 10 the patient underwent fiberoptic bronchoscopy. A right lower lobe biopsy showed an occasional noncaseating granuloma consistent with sarcoidosis. Stains for acid-fast bacteria (AFB) and fungi were negative.

A chest radiograph obtained September 10 following bronchoscopy showed a fine reticular interstitial pattern

involving the mid to lower lung. The patient was begun on a second course of prednisone and showed some improvement. A chest radiograph obtained on September 22, however, showed an interstitial lung process involving all areas, although both lungs appeared radiographically improved. Prednisone was discontinued on September 23 in anticipation of an open lung biopsy. Oxygen saturation ($SaO_2$) measurements before surgery on 4 liters of $0_2$ ranged from 88% to 92%.

On September 24, the patient had an open lung biopsy of the lingula and left lower lobe. The lingula showed moderate to severe granulomatous inflammation with AFB, numerous granulomas with focal caseation and necrosis, interstitial chronic inflammation, and mild and interstitial immature fibrosis with focal pattern. The left lower lobe of the lung showed moderate granulomatous inflammation, multiple granulomas with focal caseation and necrosis, and mild interstitial chronic inflammation and immature fibrosis. Aerobic tissue cultures showed rare gram-positive cocci. Stains and cultures for fungi and *Legionella* were negative, as were a shell vial assay and culture for cytomegalovirus. An intradermal test with 5 tuberculin units of purified protein derivative S (PPD) placed on September 24 was negative. Serologic HIV test results were also negative. A chest radiograph obtained September 25 showed bilateral bibasilar consolidation consistent with atelectasis or pneumonia with some infiltrate in the upper lobes.

On September 25, the patient was begun on oral isoniazid, 300 mg; rifampin, 600 mg; ethambutol, 400 mg; and pyrazinamide, 500 mg daily for suspected miliary tuberculosis. This regimen was discontinued 7 days later.

On subsequent evaluation at National Jewish Hospital in early October, the patient had $pO_2$ 40, $pCO_2$ 36, pH 7.43, and $SaO_2$ 77% on room air. High-resolution CT scan showed fine central lobular nodularity without bronchiectasis. Pulmonary function tests were remarkable (Table).

Case 2: The 44-year-old husband of Patient 1 was well until mid-August when he had onset of productive cough, fever, and night sweats. Over the next month, he had an 8-pound weight loss. His past medical history is remarkable only for a history of heavy smoking (1/2 to 1 pack per day) until 7 years previously, when he stopped smoking entirely.

Cases 3, 4, and 5: The 14-, 12-, and 9-year-old sons of Patients 1 and 2 became ill in mid-September with influenza-like symptoms, including fever as high as 104°F, nausea, vomiting, and shortness of breath. The 12-year-old (Patient 4) was hospitalized for dehydration. His chest radiograph showed diffusely abnormal lungs with mildly increased nodular markings more prominent in the lower than upper lobe. Pulmonary function tests were performed for Patient 4 in early October (Table).

A chest radiograph for Patient 3 showed ground-glass alveolar infiltration with tiny opacities, suggesting a miliary pattern consistent with tuberculosis. Patient 5's chest radiograph showed mild increased streaking bilaterally.

All family members had negative skin tests with PPD at 48 and 72 hours. Although none described potential exposures to tuberculosis, the prospect of a family with tuberculosis prompted retrieval of specimens and pending cultures from the local hospital. Probe tests at the state public health laboratory on these specimens and sediments of centrifuged culture medium were all negative for *M. tuberculosis* but positive for MAC for two family members. Because of these laboratory findings and the unusual occurrence of five cases of suspected pulmonary tuberculosis among family members, the cases were presented to a physician at National Jewish Hospital, who noted the possibility of NTM-related disease secondary to hot tub exposure.

### Environmental Investigation

Approximately a year earlier, the family had installed a hot tub in an enclosed sunroom next to the kitchen. The source of water for the tub was surface water from the Boulder municipal system, transported via tanker truck. Drinking and bathing water come from an alluvial aquifer well shared with several neighbors.

The hot tub water was changed only two or three times from January to October. The tub was equipped with an ozonator. On occasion a chlorine/bromine float or a cup of bleach would be added just before the tub was used. Disinfectant levels or pH were not checked.

Patient 1 used the hot tub rarely, most recently once in June and a second time in July. Because the tub water irritated her skin, she showered immediately after using the tub. However, when one of her sons was in the tub, she generally stood nearby. The 12-year-old, Patient 4, was the most frequent user. The three children often entered the tub after having been outside, without having showered first. Patients

Table. Laboratory and pulmonary function test results for five patients with nontuberculous *Mycobacterium* infection

| | Sputum tests | | Pulmonary function tests | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $FEV_1$ | | FVC | | DLCO/VA | | RV | |
| Patient | AFB[a] | MAC | initial | 6 mo | initial | 6 mo | initial | 6 mo | initial | 6 mo |
| 1 | - smear | + culture | 1.40 (42%) | 2.47 (75%) | 1.86 (44%) | 3.25 (80%) | 82% | 87% | 179% | 147% |
| 2 | + smear | + culture | | -- | | -- | | -- | | -- |
| 3 | - smear | + culture | | -- | | -- | | -- | | -- |
| 4 | + smear + culture | - probe | 1.98 (61%) | 2.63 (76%) | 2.57 (68%) | 3.41 (84%) | 91% | 98% | 226% | 88% |
| 5 | - smear - culture | -- | | -- | | -- | | -- | | -- |

[a]AFB = acid-fast bacilli; MAC = *Mycobacterium avium* complex; $FEV_1$ = forced expiratory volume; FVC = forced vital capacity; DLCO/VA = diffusing capacity of the lung for carbon monoxide; RV = residual volume; – = not done.

1 and 4 had the greatest exposure to the hot tub aerosols. In retrospect, they described a clear relationship between hot tub exposure and worsening of symptoms, i.e., recurrence of night sweats, chills, and fever.

Following identification of MAC and *M. fortuitum* from clinical specimens (Table) and further consultation, Patients 1 and 4 were begun on a regimen of rifampin, ethambutol, amikacin, clarithromycin, ciprofloxacin, and prednisone. Patients 2, 3, and 5 were treated with clarithromycin and ciprofloxacin. After 6 months, pulmonary function tests for Patients 1 and 4 had improved (Table), signs and symptoms had resolved, and chest radiographs were normal for all family members.

The results of sputum evaluation on smear and culture for AFB are summarized (Table). Patient 4 was smear and culture positive for AFB, but negative on probe for MTB or MAC. The organism was subsequently identified as *M.foruitum*.

Patient and water isolates were initially identified as MAC and typed by MEE, with identical enzyme profiles (25). Restriction fragment-length polymorphism (RFLP) analysis with an insertion sequence specific for *M. avium* (IS *1245*) (26) confirmed that all isolates were the identical strain of *M. avium* (Figure). Isolation of *M. fortuitum* from this hot tub has been described (27).

## Conclusions

NTM organisms isolated from the hot tub are likely responsible for this family's illness for the following reasons: exposure to the hot tub was temporally related to onset of symptoms; MAC was isolated from the lung biopsy and sputum of one patient and the sputum of two others, as well as from the hot tub; and MAC isolates from patient specimens and the hot tub were identical by RFLP. In addition, *M. fortuitum* was isolated from both the hot tub and a fourth hot tub-exposed person.

The source of the MAC and *M. fortuitum* is unclear. Our inability to isolate either organism from samples from the tanker truck used to supply water for the hot tub does not rule this out as a source. NTM have been isolated from municipal water supplies in the past (22). Alternatively, the users may have introduced the organisms, as the children often used the tub without showering first.

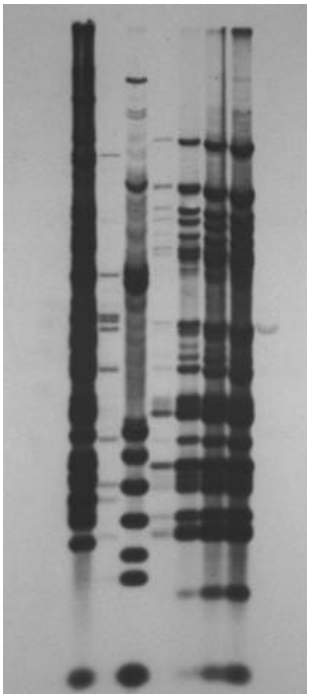

Figure. Restriction fragment-length polymorphism analysis for isolates from patients and hot tub. Lane 1: *Mycobacterium avium* CDC #91-9282, serotype 4. Lane 2: *M. avium* CDC #91-9285, serotype 10. Lane 3: *M. avium* CDC #91-9299, serotype 8. Lane 4: Hot tub isolate. Lane 5: Isolate from Patient 2. Lane 6: Isolate from Patient 3. Lane 7: Isolate from Patient 1.

Proliferation of these organisms in a hot tub is not surprising, as both MAC and *M. fortuitum* are thermophilic (12). Moreover, at temperatures >84°F, chlorine loses much of its efficacy as a disinfectant (15).

Controversy exists as to whether persons with pulmonary disease secondary to NTM are experiencing a hypersensitivity reaction to the organisms or symptoms secondary to true infection (28,29). Murphy concludes that in the presence of dyspnea, nodular infiltrates seen on CT, response to steroids, and absence of predisposing factors such as chronic lung disease, a patient with MAC-related lung disease has hypersensitivity pneumonitis. Pathologic findings, including palisaded and multinucleated histiocytes and granulomatous inflammation, however, suggest infection (28). In a recent case presentation, symptoms and radiographic findings in a patient from whose lung tissue MAC was cultured are consistent with both the diagnoses of hypersensitivity pneumonitis and atypical mycobacterial infection, a conclusion substantiated by pathologic findings (29). In cases evaluated at National Jewish Medical and Research Center, most patients required treatment with both steroids and antimycobacterial medications (30). This experience suggests that NTM disease represents a spectrum of disease with components of both hypersensitivity pneumonitis and infection.

Our cases had characteristics of both hypersensitivity pneumonitis and true infection. The short interval between hot tub use and exacerbation of symptoms and the patchy ground-glass appearance of the lungs, with centrilobular nodules on CT, suggest hypersensitivity pneumonitis (31). The granulomas seen on pathologic examination and the response to treatment with antimycobacterial medications, however, suggest true infection. The temporary improvement in Patient 1's condition after she received prednisone may represent either appropriate treatment of hypersensitivity pneumonitis or a decrease in granulomatous inflammation in the bronchioles, secondary to infection (28).

Little data exist to explain the mechanism of disease caused by NTM in healthy persons. Exposure to sufficiently large and repeated inocula of the organism in droplets of readily respirable size appears to be sufficient to overwhelm normal host defenses.

Hot tubs should be maintained according to manufacturers' recommendations, which include both frequent water changes and adequate use of disinfectants. In addition, placing a hot tub in an enclosed environment should be strongly discouraged. Patients with atypical pneumonia should be questioned about similar illnesses among family member and others who have had similar exposures, including exposure to a hot tub. As hot tubs become increasingly popular (pers. comm., John J. Cergol, Jr.), hot tub-related illness associated with NTM may become an emerging infectious disease challenge.

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# Catheter-Related Bacteremia due to *Streptomyces* in a Patient Receiving Holistic Infusions

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*Streptomyces* species are rare causes of invasive infection in humans. We report the first documented case of a catheter-associated bacteremia due to *Streptomyces*. The most likely source of infection was unlicensed, injectable holistic preparations that the patient had received. We review reported cases of invasive infections caused by *Streptomyces* and comment on the potential infectious complications of parenteral holistic treatments.

*Streptomyces* species are aerobic actinomycetes best known for their production of antimicrobial substances. They infrequently cause human disease, most often manifesting as a localized, chronic suppurative infection of the skin and underlying soft tissue (1). Nonmycetomic infections caused by *Streptomyces* species are very rare.

We report the first documented case of a catheter-associated bacteremia caused by *Streptomyces*. The most likely source of infection was unlicensed, injectable holistic preparations that the patient had received. Identifying the use of alternative medicines by patients may be essential in evaluating cases or outbreaks of new or unusual organisms.

## Case Report

A 49-year-old African-American woman had a 10-year history of breast cancer, for which she had declined chemotherapy; she opted to receive holistic treatments only. Her disease progressed; she underwent bilateral mastectomies and bone metastases developed. Approximately 1 year before admission, the patient had a subcutaneous central venous catheter placed to receive intravenous infusions of holistic preparations.

She reportedly received infusions of glutathion, germanium, superoxide dismutase, interferon (100,000 IU), manganese, selenium, zinc, magnesium chloride, calcium gluconate, potassium chloride, and vitamin C (25 g), as well as "NeyTumorin," a holistic preparation (2).

Three weeks before admission, the patient complained of feeling feverish after receiving an infusion at her provider's office. Her temperature was 38°C. Blood drawn through the subcutaneous catheter was sent for culture and was reported as growing "diphtheroids." Eleven days later, repeat blood cultures drawn through the catheter grew gram-positive bacilli, which a laboratory could not identify further. At that time the patient was referred to our hospital for admission.

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The patient's medications at the time of admission were pamidronate, a multivitamin, and the holistic infusions. She had no history of alcohol or illicit drug use. She denied HIV risk factors but had never been tested for HIV. She had not traveled recently and had no remarkable animal exposures. She had previously worked as a nurse.

On examination, she appeared nontoxic; her blood pressure was 143/83 mmHg, heart rate was 84 beats per minute, respiratory rate was 16, and temperature was 37.3°C. Physical examination revealed a subcutaneous port in her right anterior chest, which showed no signs of infection; well-healed bilateral mastectomy scars; and a grade II/VI systolic heart murmur.

After blood had been drawn for cultures, the patient was started empirically on vancomycin. Blood drawn peripherally and through the subcutaneous catheter on hospital days 1, 2, and 3 grew gram-positive elongated bacilli in the aerobic culture bottles. Her central venous catheter was removed on the fourth hospital day. Culture of the catheter tip was negative. A transthoracic echocardiogram showed no valvular vegetations.

When examined directly from liquid media, the organism appeared as long, branching, beaded, gram-positive bacilli. However, the organism grown on agar plates appeared more fragmented, as single, gram-positive bacilli. The isolate was catalase positive and modified acid-fast negative; aerial mycelia were present. There was no growth in lysozyme. The organism also hydrolyzed casein, xanthine, and tyrosine and yielded positive starch, gelatin, nitrate, and esculin reactions. Our laboratory identified the organism as a *Streptomyces* species. This isolate was sent to the Centers for Disease Control and Prevention (CDC) for confirmation of identification, and cell-wall analysis identified the peptidoglycan L-diaminopimelic acid, confirming that the isolate was a member of the genus *Streptomyces* (3).

The antibiotic regimen used to treat the patient was changed to intravenous ceftriaxone, 1 g every 24 hours, and oral clarithromycin, 500 mg twice a day. Her fever resolved, and she remained asymptomatic; repeat blood cultures were negative. A percutaneous indwelling central catheter was placed, and the patient was discharged to home with

recommendations to complete a 1-month course of ceftriaxone and a 6-month course of clarithromycin.

Three months after hospital discharge, the patient had no evidence of residual infection, and repeat blood cultures were negative. She was subsequently admitted to the palliative care unit for management of pain due to diffuse bone metastases and eventually died.

## Conclusions

The most common manifestation of *Streptomyces* infection, mycetoma, typically results from inoculation of the microorganism through an injury caused by a thorn and usually involves the legs and feet (4). Invasive *Streptomyces* infections are extremely rare. A Medline search from 1966 to 2000 identified only 10 cases of invasive *Streptomyces* infection, defined as infection other than mycetoma or superficial skin infections (1,5); in none of the cases was infection caused by catheter-associated sepsis. Previous cases included four cases of pneumonia and one each of lymphadenitis, pericarditis, brain abscess, peritonitis, endocarditis, and intraspinal mycetoma. Five of the patients had underlying conditions identified: four were HIV infected, and one had a prosthetic aortic valve. In only two of the nine cases (one of pneumonia and one of endocarditis) was *Streptomyces* cultured from the blood.

Treatment recommendations for *Streptomyces* infections are generally based on in vitro data and analogies from data on the treatment of *Nocardia* infections (1). McNeil et al. performed in vitro tests of the susceptibility of aerobic actinomycetes species, including 28 isolates of *S. griseus* referred to CDC from October 1985 to February 1988 (6). These data suggest that the best antimicrobial options for visceral *Streptomyces* infection include macrolides, minocycline, doxycycline, ceftriaxone, and imipenem (6). Twenty-nine percent of *S. griseus* species tested at CDC were resistant to trimethoprim/sulfamethoxazole, the drug of choice for treatment of *Nocardia* infections (6).

A variety of antimicrobial regimens were used in the previously reported cases, and in most the outcome was good, with resolution of infection. However, the optimal choice of antimicrobial agent and duration of therapy for *Streptomyces* visceral infections remain to be determined.

Our patient had clearance of bacteremia after removal of the indwelling central catheter and a course of ceftriaxone and clarithromycin. While the catheter tip culture was negative and hence an alternate focus cannot be entirely excluded, the patient's fever resolved and blood cultures promptly cleared after catheter removal, supporting the inference that the infection was catheter associated.

The previous reports of invasive *Streptomyces* disease do not mention putative sources of the infections. For our patient, who had no history of travel or unusual agricultural exposures, a possible source of infection was the unlicensed, injectable holistic preparations that she had received. Attempts to obtain samples of the intravenous holistic preparations for culture were unsuccessful, as were attempts to assess whether other patients had received this preparation and whether any other infectious complications had occurred. Information on any measures used to ensure product sterility was unavailable.

Three times a week, our patient was receiving infusions of a preparation that included a compound called NeyTumorin, which reportedly consists of "a combination of peptides and proteins of 15 different organs from fetal and young pigs or cows" (2). The inventor of NeyTumorin claims that "physiologic repair aids" from the cytoplasm of healthy animal organs result in immunogenic and immunomodulatory effects that improve a patient's biologic response to malignancy (2). However, the specific components of NeyTumorin are not defined, the claimed mechanism of action has not been proven, and there is no evidence of clinical efficacy. Clinical studies are described as inconclusive "because of false or insufficient documentation" (2).

The use of alternative and herbal medicines in the United States has increased steadily over the past decade (7,8). Patients frequently believe that if a substance is "natural," it is safe; likewise, providers often assume that such substances are unlikely to be harmful (9). However, recent data have demonstrated that some holistic medicines may result in a variety of undesirable effects. Acute rejection has been reported in two heart transplant patients due to a metabolic interaction between cyclosporine and St. John's wort (*Hypericum perforatum*), which induces the 3A4 isoform of the cytochrome P450 enzyme system (10). Through a similar drug-to-drug interaction, St. John's wort has been shown to lower plasma levels of the protease inhibitor indinavir, potentially placing patients at risk for antiretroviral resistance and treatment failure (9).

Alternative remedies may also directly cause adverse drug effects; interfere with laboratory assays; contain unrecognized, potentially harmful contaminants; and, if nonsterile, transmit infections (9-11). Distribution of an unlicensed injectable preparation, purported to contain adrenal cortex extract contaminated with *Mycobacterium abscessus*, led to a multistate outbreak of soft-tissue abscesses (11). Our case may represent another instance of an infection transmitted by the injectable use of a holistic preparation.

To create an environment that is conducive to open communication and education, physicians should discuss alternative therapies nonjudgmentally with patients to identify what substances patients may be using (7). Given the increased use of alternative medicines, including those administered parenterally, increased vigilance in monitoring patients for potential infectious complications of such treatments is needed. Clinicians should be aware of data addressing the safety and efficacy of alternative and herbal preparations and of reliable data sources such as the National Institutes of Health's National Center for Complementary and Alternative Medicine (nccam.nih.gov) and the Office of Dietary Supplements (odp.od.nih.gov/ods).

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# A Multistate Outbreak of *Salmonella enterica* Serotype Baildon Associated with Domestic Raw Tomatoes

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*Salmonella enterica* serotype Baildon, a rare serotype, was recovered from 86 persons in eight states; 87% of illnesses began during a 3-week period ending January 9, 1999. Raw restaurant-prepared tomatoes were implicated in multiple case-control studies. Contamination likely occurred on the farm or during packing; more effective disinfection and prevention strategies are needed.

We report our investigation of a large, multistate outbreak of 86 cases of salmonellosis associated with eating raw, domestic tomatoes; this is the third such outbreak in the United States in recent years (1,2).

## The Study

Outbreak patients were persons from whom *Salmonella enterica* serotype Baildon was recovered between December 1, 1998, and March 1, 1999. *S*. Baildon is rare; only five isolates were reported nationwide in 1997 (3). To increase case finding, the Centers for Disease Control and Prevention (CDC) notified epidemiologists and public health laboratorians nationwide about the outbreak.

After hypotheses-generating interviews in three states, patients from Arizona, California, Georgia, and Virginia were enrolled in four independently conducted case-control studies. Each study explored food items eaten, and place of food preparation and consumption (home vs. institution or restaurant), for the 5 to 7 days before illness began. Controls were matched to patients by gender, age, geographic area, and case-specific exposure period. Ten Arizona patients were compared with 18 controls identified by systematic telephone digit-dialing. Seventeen California patients were compared with 32 healthy controls previously infected with nontyphoidal *Salmonella*; five Georgia patients were compared with 10 controls identified by patients as friends; 11 Virginia patients were compared with 33 controls drawn from a systematic sample of reverse telephone directories.

The distribution sources of tomatoes for 15 tomato point of service (POS) exposures reported by 14 patients in California and Virginia were examined. POS included three Virginia and two California restaurants, six outlets of one Mexican fast-food restaurant chain in California, and two Virginia nursing homes. Tomato operations were observed at one grower/packer cooperative, five Virginia facilities, and the sole-source processor of diced tomatoes used by the Mexican restaurant chain in California.

We calculated Mantel-Haenszel matched odds ratios and p values to assess univariate associations between food items and illness. Three of four case-control studies suggested two food items. Using data collected from the California case-control study, we assessed the independent association of these two food items by conditional logistic regression.

We identified 86 patients from eight states (Table). Onset dates were from December 6, 1998 to February 2, 1999; 87% occurred in a 3-week period ending January 9, 1999. Three elderly persons died.

Arizona patients were significantly more likely than controls to report eating at a specific chain of Mexican fast-food restaurants (60% vs. 13%, matched odds ratio (MOR) undefined, p = 0.008) but no food item was implicated. California patients were significantly more likely than controls to report eating five restaurant-prepared foods: raw tomatoes (94% vs. 33%, MOR 20, p = 0.002), iceberg lettuce (88% vs. 40%, MOR 16.5, p = 0.008), cheese (88% vs. 43%, MOR 6.6, p=0.02), raw onions (77% vs. 16%, MOR 10.3, p<0.001) and sour cream (76% vs. 11%, MOR 21.5, p <0.001). California patients were also more likely than controls to report eating at the same chain of Mexican fast-food restaurants identified in the Arizona study (63% vs. 17%, MOR 14.5, p = 0.01). There was a trend toward an association with eating home-prepared raw tomatoes (81% vs. 48%, MOR 6.0, p = 0.08). In a regression model containing both restaurant-prepared tomatoes and iceberg lettuce, tomatoes but not iceberg lettuce remained associated with disease (maximum likelihood estimate [MLE] 11.2, Wald p = .08 vs. MLE 1.6, Wald p = 0.74).

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Table. Characteristics of patients with culture-confirmed *Salmonella enterica* Serotype Baildon

| State | Cases (#) | Hospitalized[a] (#) | Deaths[a] (#) | Median age (years) | Age range (years) | Patients ≥18 years of age (%) | Female (%) | Range of onset dates[b] |
|---|---|---|---|---|---|---|---|---|
| CA | 44 | 11 | 1 | 33 | <1-82 | 89 | 65 | 12/18/98-02/02/99 |
| VA | 13 | 4 | 1 | 47 | 20-86 | 100 | 69 | 12/21/98-01/09/99 |
| AZ | 13 | 0 | 0 | 26 | 18-69 | 92 | 69 | 12/18/98-01/29/99 |
| GA | 8 | 1 | 1 | 38 | 17-86 | 88 | 75 | 12/19/98-02/02/99 |
| IL | 3 | 0 | 0 | 43 | 32-58 | 100 | 33 | 12/23/98-01/07/99 |
| AL | 2 | 0 | 0 | 66 | 45-86 | 100 | 100 | 01/07/99 |
| TN | 2 | 0 | 0 | 46 | 41-51 | 100 | 50 | 01/04/99-01/06/99 |
| KS | 1 | 0 | 0 | 22 | 22 | 100 | 100 | 12/06/98 |
| Total | 86 | 16 | 3 | 35 | <1-86 | 93 | 67 | 12/06/98-02/02/99 |

[a]Data available for 91% (78) of patients.

[b]Specimen collection date was used if patient's symptom onset date was unknown (n = 10)

Georgia patients demonstrated a trend toward eating restaurant-prepared tomatoes (80% vs. 20%, MOR 8.7, p = 0.09) and iceberg lettuce (100% vs. 60%, MOR undefined, p = 0.10). Virginia patients were significantly more likely than controls to report eating institution- or restaurant-prepared raw tomatoes (91% vs. 45%, MOR 11.1, p = 0.009) and cucumbers (73% vs. 33%, MOR 5.4, p = 0.03). Patients demonstrated a trend toward eating restaurant-prepared iceberg lettuce (73% vs. 52%, MOR 2.2, p = 0.21), raw onion (55% vs. 27%, MOR 2.9, p = 0.1), and romaine lettuce (36% vs. 9%, MOR 4, p = 0.07).

The traceback identified two tomato grower/packer cooperatives, in Florida, which could have supplied tomatoes eaten by the 14 patients who reported only one or two POS encounters during the exposure period. In April 1999, the only cooperative still packing tomatoes was investigated. Tomatoes had reportedly been hand-picked and were transported to the packing facilities in covered bins. Tomatoes were unloaded into a dump tank and moved by a flume system (water temperature 38.7°C, pH 6.5, target chlorine reported as 125 ppm but not measured) to a warm spray wash. Tomatoes were mechanically sorted (unacceptable tomatoes were manually removed), waxed, and boxed. Packed tomatoes were stored at 21.1°C in ripening rooms.

The tomato dicing operation in California was inspected in May 1999. Uncored tomatoes were washed, inspected for decay, color, and stem removal, and then conveyed to a mechanical dicer. Diced tomatoes were moved by a flume system to a perforated shaker-belt conveyor, mechanically packaged into 5-pound trays, sealed and stored at 4.4°C. Tomatoes were held for one day before being shipped by refrigerated truck to two distributors. Target water temperature, total chlorine, and hold-times for the bath and flume were reported by the processor as 1.1°C, 100-130 ppm, and 1-2 minutes, respectively. Wash water temperatures and chlorine levels were maintained manually whereas the flume system was chlorinated by an automated system. During inspection, this system's pH monitor did not work. Temperature was measured at 2.20°C.

Tomatoes served in Virginia were processed at the individual POS facilities. Whole, uncored tomatoes were washed and cut by knife or mechanical chopper.

## Conclusions

We report on a large, multistate outbreak caused by *S.* Baildon, an unusual *Salmonella* serotype. The outbreak was associated with eating raw tomatoes. Because less than three percent of estimated *Salmonella* cases are officially reported nationwide (4,5), this outbreak could have included 3,300 cases.

Raw tomatoes were epidemiologically implicated as the source of this outbreak. This finding is supported by several observations. First, eating raw tomatoes was strongly associated with illness in the case-control studies, and nearly all patients ate them. Second, these studies were conducted independently, using different control recruitment strategies. Third, raw tomatoes have a 3-week shelf life, consistent with the brief occurrence of the outbreak.

That many restaurants across several states were involved suggests the tomatoes were likely contaminated early on—at the farm or during packing. Salmonellae can grow on tomato skin surfaces and infiltrate core tissues during tomato harvest, packing, and transportation (6,7). Air spaces in tomatoes at high field-heat temperatures can constrict when submerged in cool water. As air space volume decreases, water and salmonellae can be drawn (by vacuum effect) from the dump tank into the fruit through the stem scar. For these reasons, postharvest process water should be potable and warmer than the incoming fruit (8).

Once tomatoes are contaminated, elimination of salmonellae can be difficult. While chlorine levels of 200-250 ppm would be expected to substantially reduce salmonellae (6,7), even higher levels of chlorine disinfection (320 ppm) did not eliminate salmonellae from tomatoes in one laboratory study (6). The efficacy of chlorine against salmonellae depends, in part, on the location and amount of contamination. Salmonellae inoculated onto stem scars and growth cracks survived disinfection better than on smooth tomato skins (7).

The grower/packer cooperative we observed had at least some elements of a hazard analysis critical control point (HACCP) program for commercial tomato packinghouses (9) including warm, chlorinated wash water. However, we observed operations after the outbreak and did not have access to historic water quality measures (free chlorine, pH, and temperature). Even if free chlorine levels of 125 ppm

were maintained, such levels would not be expected to eliminate organisms in stem scars or damaged tomato skin.

Dicing and pooling of contaminated tomatoes in our outbreak may have played a role in amplifying the amount of contaminated product, just as these were suspected to have played a role in prior outbreaks (2). The diced tomato processor we observed in California exposed both whole and diced tomatoes to chlorine. However, laboratory experiments demonstrated that *S.* Baildon could survive disinfection with 200 ppm chlorine in diced tomatoes (10). Microorganisms in tomatoes are highest around the stem scar and central core (11), where they are less accessible to chlorine (7). Therefore, the practice of including stem scars and cores in pooled, finished product could have increased the opportunity for amplification, especially if the diced tomatoes were later mishandled. Contamination of internal tissue from the outer skin and stem scar can also occur during cutting and slicing (12). Numerous *Salmonella* serotypes, including our outbreak strain, grow rapidly in cut tomatoes held at room temperature (6,7,10,13). If the involved restaurants maintained tomatoes at room temperature for extended periods, even small populations of salmonellae on sliced or diced tomatoes could have grown rapidly.

While chlorine-based water quality systems may markedly reduce salmonellae contamination, they cannot be relied upon to eliminate it. A terminal treatment step with demonstrated effectiveness against *Salmonella*, such as irradiation (14,15), should be considered, particularly since tomatoes are commonly eaten raw and have now been implicated in three multistate outbreaks.

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Ms. Cummings is an epidemiologist in the California Department of Health Services; her work focuses on the epidemiology of infectious diseases in California.

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# Contact with Farming Environment as a Major Risk Factor for Shiga Toxin (Vero Cytotoxin)-Producing *Escherichia coli* O157 Infection in Humans

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In a prospective, unmatched case-control study of sporadic Shiga toxin (Vero cytotoxin)-producing *Escherichia coli* O157 (STEC O157) infection in England, exposure to the farming environment emerged strongly as a risk factor (adjusted odds ratio = 2.45; 95% confidence intervals = 1.49-4.02; p=0.0004) posing further challenges and opportunities for prevention.

Shiga toxin (Vero cytotoxin)-producing *Escherichia coli* O157 (STEC O157) is an important emerging pathogen worldwide, and the illness and death associated with infection are considerable (1). Outbreaks of STEC O157 have been attributed to consuming contaminated food (especially undercooked ground beef) and water, animal contact, and person-to-person transmission (2,3). However, sporadic infection accounts for approximately 80% of all STEC O157 diagnosed in England and Wales (3). Therefore, the sources of and routes of transmission for most infections remain largely unknown. We report the results of a prospective unmatched case-control study, undertaken in England from October 1996 through December 1997. The aim was to identify risk factors for sporadic STEC O157 infection.

## The Study

A patient was defined as a person with abdominal pain or diarrhea (three or more loose stools in a 24-hour period) from whom STEC O157 had been isolated by fecal culture at any of the 47 Public Health Laboratory Service (PHLS) laboratories in England. Patients were included if they were the index patient in the household, normally resided in England, had not traveled abroad in the 5 days before the onset of symptoms, were not part of a known outbreak, and had no evidence of mixed infection. The study took place from October 1, 1996, through December 31, 1997. Ethical approval was obtained from the PHLS Ethics Committee.

A local study coordinator reported positive laboratory results to a central study coordinator at the PHLS Communicable Disease Surveillance Centre (CDSC), complete with details of each patient's general practitioner (GP). The patients' GPs nominated up to three asymptomatic community controls, selected on the basis of gender and age group, for each patient. A standard, structured questionnaire was posted to each study subject along with a reply-paid evelope. The 15-page questionnaire covered demographic and clinical details and food, water, occupational, recreational, and household exposures in the 5 days before the patient's date of onset. Most items were close-ended questions. Nonresponders were sent a second mailing. The data returned to CDSC were entered onto an Epi-Info database (4) and validated by means of double data entry.

Single-risk variable analysis was undertaken by calculating odds ratios and 95% confidence intervals and by continuity-corrected chi-square tests. Variables associated with illness at the 10% significance level in the single-risk variable analysis were included in a logistic regression model. A 10%, rather than the standard 20%, significance level was used because of the large number of variables considered (n=43). Season (October-March and April-September), age group ($\leq$5 years, 6-19, 20-59, and $\geq$60), and gender were included in the model. Terms were assessed by comparing nested models using likelihood ratio tests. Those not reaching a 10% significance level were subsequently rejected from the model. Analyses were performed by using SAS (SAS Institute Inc., Cary, NC) and GLIM (5).

Data were obtained for 369 patients (response rate = 84%) and 511 controls (response rate = 57%). The male-to-female ratio for patients was 1:1. There were, however, slightly more female controls (55.5%) than males. The age range of patients was 2 months to 84 years. Controls were slightly older than patients (median age 21 years for controls, 17 years for patients). Forty-one percent (150/369) of patients were <10 years of age, and 27% (100/369) were <5 years. Sixty-two percent of patients (228/369) had bloody diarrhea, and 38% (140/369) were admitted to hospital.

The risk of developing STEC O157 infection was strongly associated with contact with the farm environment (Table). This encompassed recreational visits by members of the public to open farms (petting zoos) or staying on farms for their holidays (e.g., in farm cottages), and work-related visits to farms. The last category comprised workers (e.g., electricians, maintenance engineers, delivery drivers) who entered farm premises for work purposes but who did not

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Table. Risk factors for sporadic cases of Vero cytotoxin-producing *Escherichia coli* O157 infection in England: logistic regression analysis

| Variable[a] | Adjusted odds ratio | 95% CI | p value | No. (%) patients exposed in single-risk variable analysis | No. (%) controls exposed in single-risk variable analysis |
|---|---|---|---|---|---|
| Rare chicken | 5.13 | 1.44, 18.26 | 0.009 | 16 (4.7) | 6 (1.3) |
| Purchasing food from a market stall | 2.93 | 1.22, 7.07 | 0.02 | 25 (7) | 17 (3.6) |
| Watercress | 2.61 | 1.24, 5.47 | 0.01 | 29 (8.9) | 23 (4.9) |
| Farm contact | 2.45 | 1.49, 4.02 | 0.0004 | 87 (23.6) | 62 (12.9) |
| Travel (nights away from home) | 2.23 | 1.35, 3.71 | 0.002 | 100 (27.3) | 53 (11.2) |
| Paddling (wading) | 2.13 | 1.04, 4.35 | 0.04 | 40 (11.2) | 24 (5.1) |
| Peaches | 2.08 | 1.17, 3.72 | 0.01 | 53 (15.8) | 37 (7.9) |
| Drank pasteurized milk | 0.66 | 0.43, 1.01 | 0.06 | 240 (68.2) | 351 (74.4) |
| Bought frozen meat | 0.63 | 0.43, 0.95 | 0.03 | 225 (62.5) | 345 (73.6) |
| Ate butter | 0.56 | 0.38, 0.82 | 0.003 | 175 (48.9) | 278 (59.1) |
| Consumed cream | 0.43 | 0.26, 0.7 | 0.0005 | 61 (16.9) | 133 (28.5) |

[a]This model was adjusted for season, age group, and gender and was based on 607 (80%) of the observations (losses due to missing data for one or more of the explanatory variables).
CI = confidence intervals.

regularly work in the farming environment. With respect to recreational visits, approximately half the patients exposed in the single-risk variable analysis reported touching farm animals. The remainder had simply been exposed to the environment. Farmers who routinely worked with livestock were not found to be at increased risk.

Travel away from home during the exposure period was also associated with increased risk for infection. Of those who had spent nights away from home, most (87%) had traveled elsewhere in the United Kingdom as opposed to staying with friends or relatives locally.

Although eating rare chicken and watercress and purchasing food from a market stall were associated with increased risk for STEC O157 infection, these exposures accounted for a very small proportion of patients in the single-risk variable analysis. Consumption of cream and butter and purchasing frozen meat were inversely associated with risk for STEC O157 infection. Eating ground beef was not associated with infection in this study.

## Conclusions

Contact with the farming environment, which included recreational or occupational visits, was strongly associated with sporadic STEC O157 infection in England. The risk occurred in people not routinely exposed to the farming environment, i.e., members of the public visiting open farms or spending holidays on farms, or people who had recently gone onto a farm for work but who were not regularly employed on farms. In contrast with recreational visits, for the work-related visits we were unable to differentiate between animal contact and simply spending time in the farm environment. Although farmers were not found to be at increased risk for infection with STEC O157, we were unable to determine the risk among farmers' children since the questionnaire sought only occupational details and the address information was insufficient to allow us to determine farming premises with accuracy.

We performed an unmatched prospective case-control study using self-administered questionnaires because this design permits efficient study of large numbers of patients and controls. However, we must consider the sources of bias. Patients were recruited through the PHLS national network. We did not include cases diagnosed in National Health Service (NHS) laboratories in order to reduce the opportunity for selection bias based on diagnostic criteria. Since 1995 it has been PHLS policy for all laboratories in the network to test all diarrheal stools by standard protocols (6). Many non-PHLS laboratories appear to use more selective screening protocols, e.g., testing samples from infants and the elderly and samples containing frankly bloody stools. Including cases from NHS sources, therefore, would have favored the selection of infants, the elderly, or those with more severe symptoms. Although this means of patient recruitment might be considered to limit the representativeness of the study, the fact that most cases of STEC O157 in England were diagnosed by the PHLS during the study mitigates this concern.

Matching was not used, the danger being that the patient and control populations might have been systematically different. However, the recruitment of controls through the patients' GPs ensured that controls were drawn from the same population as the patients. Furthermore, the potential confounders of age and gender were included as variables in the logistic regression analysis.

Direct zoonotic and environmental transmission have emerged as important risk factors for outbreaks of STEC O157 in the United Kingdom in recent years (2,7,8). Our results suggest, however, that for sporadic cases of STEC O157, transmission of infection directly from the farm environment to humans appears to be more important than is

generally recognized. This means that the patient history for STEC O157 infection and other potentially zoonotic diseases should routinely include a determination of exposure to farm animals or the farm environment.

Our findings are consistent with previous descriptive studies undertaken in Scotland (9) and the southwest of England (10) and the results from a case-control study in Wales (11). These findings indicate opportunities for prevention. People aware of the risks associated with this exposure are empowered to take simple measures to prevent themselves from becoming infected, such as washing their hands after coming into contact with livestock or farm animal feces.

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# Rift Valley Fever Outbreak, Mauritania, 1998: Seroepidemiologic, Virologic, Entomologic, and Zoologic Investigations

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A Rift Valley fever outbreak occurred in Mauritania in 1998. Seroepidemiologic and virologic investigation showed active circulation of the *Rift Valley fever virus*, with 13 strains isolated, and 16% (range 1.5%-38%) immunoglobulin (Ig) M-positivity in sera from 90 humans and 343 animals (sheep, goats, camels, cattle, and donkeys). One human case was fatal.

In 1998, a Rift Valley fever outbreak was identified in Aioun El Atrouss, Hodh El Gharbi Region, Mauritania. This viral anthropozoonosis is transmitted by mosquitoes; it causes abortion in animals and illness ranging from febrile syndrome to hemorrhagic fever and death in humans (1). In 1987, following dam construction on the Senegal River, a major epidemic, with 200 human deaths, occurred for the first time in Mauritania (2). Since then, several smaller outbreaks have been reported, and regular circulation of the virus among cattle has been documented (3,4). We report laboratory and field investigations among animals and humans during the 1998 outbreak.

## The Outbreak

In September 1998, several patients with fever and hemorrhagic syndrome were admitted to the Hospital of Aioun El Atrouss, Hodh El Gharbi Region, Mauritania. Sera from two of these four patients were positive for *Rift Valley fever virus* (RVFV) by immunoglobulin (Ig) M detection by enzyme-linked immunosorbent assay (ELISA), virus isolation on cell cultures, and reverse transcription polymerase chain reaction, focusing on the S segment of the viral genome.

From October to the end of December, three epidemiologic investigations were undertaken in five localities in the Hodh El Gharbi region (Figure). The Hodh El Gharbi is an extensive livestock farming region in an arid area. In September 1998, rainfalls were exceptionally heavy, with a threefold increase over the 10-year average rainfall (86 mm vs. 26 mm).

During the investigations, serum samples were obtained from suspected cases in humans and animals (camels, goats, sheep, cattle, and donkeys) (Table). A suspected human RVFV case was defined as illness in any patient with fever (whether or not it was associated with hemorrhagic signs, icterus, or neurologic signs) occurring after September 1, 1998. Animal specimens were obtained from flocks in which abortions or stillbirths were reported in 1998. Based on a questionnaire among livestock breeders, the perinatal mortality rate (PMR) in flocks was estimated by using the ratio number of abortions, stillbirths, or deaths within 48 hours/number of females. Mosquitoes, sandflies, and biting midges were caught in CDC light traps. Mosquito larvae were also collected. Arthropods were sorted, pooled either by species and sex (for mosquitoes) or in polyspecific batches (sandflies, biting midges) in the field, and stored in liquid nitrogen. In light of the report on the possible involvement of rodents in


Figure. Map of Hodh el Gharbi region, Mauritania.

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Table. Serologic, virologic, entomologic, and zoologic investigations, Rift Valley fever outbreak, Mauritania, 1998

**A. Seroepidemiologic and virologic results for humans and animals**

| | No. of samples | Recent infection | Past infection | Virus isolation |
|---|---|---|---|---|
| Humans | 90 | 16.7 (15/90) | 24.4% (22/90) | 2 |

| | | Flocks investigated | | | Animals tested | | | |
|---|---|---|---|---|---|---|---|---|
| Animal species | No. of females | NA[a] | PMR (%) | CI | Median age (years) | IgM isolation | IgG alone | Virus isolation |
| Sheep | 381 | 37 | 9.7 | 0, 20.7 | 1.5 | 34.8% (31/89) | 12.4% (11/89) | 6 |
| Goats | 471 | 223 | 47.4 | 33.3, 61.4 | 3.5 | 16.3% (23/141) | 24.8% (34/141) | 5 |
| Camels | 286 | 59 | 20.6 | 1.8, 39.4 | 7.5 | 2.6% (1/39) | 0% (0/39) | 0 |
| Cattle | 36 | 17 | 4.6 | 0, 9.3 | 2.5 | 1.5% (1.69) | 33.3% (23/69) | 0 |
| Donkeys | -- | -- | -- | -- | | 0% (0/5) | 20.0% (1/5) | 0 |

**B. Entomologic results**

| Mosquito species | No. | Abundance[b] | Virus isolation |
|---|---|---|---|
| *Anopheles pharoensis* | 8 | 1.5 | 0 |
| *Anopheles rhodesiensis* | 27 | 4.9 | 0 |
| *Anopheles rufipes* | 11 | 2.0 | 0 |
| *Culex antennatus* | 1 | 0.2 | 0 |
| *Culex decens* | 25 | 4.6 | 0 |
| *Culex neavei* | 68 | 12.5 | 0 |
| *Culex perfuscus* | 4 | 0.7 | 0 |
| *Culex poicilipes* | 191 | 34.9 | 0 |
| *Culex quinquefasciatus* | 211 | 38.6 | 0 |
| Total no. of mosquitoes | 546 | 100 | 0 |
| Sandflies | 524 | | 0 |
| Biting midges | 78 | | 0 |
| Total no. of arthropods | 1,148 | | 0 |

[a]NA = Number of abortions; PMR = perinatal mortality rate; CI = 95% confidence intervals.

[b]Abundance = number of individuals of one species/total number of mosquitoes collected.

the RVFV transmission cycle in South Africa (5), wild rodents were trapped concomitantly with the arthropods. Human and animal samples were tested for RVFV-specific IgG and IgM antibodies by ELISA as described previously (6) and for virus isolation in cell cultures and suckling mice. Viruses isolated were identified by indirect immunofluorescence with RVFV-specific monovalent hyperimmune mouse ascitic fluids, as well as complement fixation and seroneutralization tests (7). Arthropods were tested for the presence of RVFV by inoculating vector suspensions into cell culture, followed by identification as described for serum samples. Pooled rodent viscera were homogenized and inoculated intracerebrally into suckling mice for virus isolation.

Among the 90 human sera tested, 16.7% had evidence of recent (presence of IgM antibody or isolated virus) and 24.4% of past (presence of IgG antibody only) infection. Two virus strains were isolated. Among the 15 recently infected patients, median age was 26 years (range 10 to 45 years), male:female ratio was 2:0, and one death was reported. Proportions of hemorrhagic signs did not differ significantly among laboratory-positive and-negative cases (40.0% vs. 28.8%; p=0.5), suggesting that a cause other than RVFV should be considered to explain hemorrhages. Conversely, the prevalence of icterus and neurologic signs was significantly higher among positive cases (46.7% vs. 19.2%, p=0.04, and 53.3% vs. 20.5%, p=0.02, respectively), suggesting that these two signs were more specific indicators of RVFV in this human outbreak.

Among animals, 343 sera were tested from five species (sheep, goats, camels, cattle, and a donkey). Except for the donkey, all the species screened were positive for IgM antibodies, with prevalences ranging from 1.5% to 34.8%. These findings indicate not only widespread circulation of RVFV in the area but are also consistent with the high perinatal mortality observed in flocks, particularly among goats. The most affected species were sheep and goats, with an IgM prevalence of 34.8% and 16.3%, respectively; moreover, 11 RVFV strains were isolated from these two species (Table). However, the discrepancy between the perinatal mortality rates and the IgM-RVFV prevalence might suggest that other diseases causing abortion may have cocirculated in the area.

Among adult mosquitoes collected, *Culex* and *Anopheles* species were the most abundant. No RVFV strain was isolated, probably because the mosquito captures were

undertaken at the end of the rainy season, when most breeding sites had dried up. This hypothesis is further strengthened by the small number of mosquitoes caught (546) and the absence of *Aedes* mosquitoes, a vector species for RVFV transmission in West Africa (8).

Seventy-three rodents belonging to five genera (*Gerbillus*, *Desmodilliscus*, *Acomys*, *Arvicanthis*, and *Jaculus*) and three families (*Gerbillidae*, *Muridae*, and *Dipodidae*) were captured in the same areas where mosquitoes were trapped, but no RVFV strains could be isolated and no serum was positive for IgM or IgG antibodies.

## Conclusions

An outbreak of Rift Valley fever occurred in the Hodh El Gharbi region of Mauritania in 1998. Because of the proportion of hemorrhagic signs in humans and the high rate of perinatal mortality among some animals, it cannot be ruled out that some other pathogen may have been involved in the outbreak; this hypothesis merits further investigation.

Since the 1987 epidemics, RVFV circulation among livestock has been documented in this region (3,4). However, outbreaks among humans and animals have probably been underreported, emphasizing the need to strengthen surveillance in the southern area of the country to prevent the potential spread of any epidemic focus to neighboring countries through nomadic animal husbandry.

Furthermore, the unique ecologic and environmental context makes the Hodh El Gharbi region of interest for research to further understand of factors influencing the emergence of this disease in West Africa.

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# Malaria in Illegal Chinese Immigrants, Italy

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A cluster of 22 imported malaria cases, 21 caused by *Plasmodium falciparum*, was observed among illegal Chinese immigrants in northern Italy in the summer of 2000. The rate of severe disease was high because the patients were not immune and they sought health-care services late in their illness because of their clandestine status. Recognition of the outbreak was delayed because no regional alert system among infectious diseases hospitals was in place.

The recent increase of population movements is paralleled by an increase in imported malaria cases in Europe, where malaria is not endemic (1,2). In Italy, migrants and foreign-born people visiting relatives represent an increasing proportion of imported malaria cases (3). Most cases are in persons from malaria-endemic areas. They rarely develop complications because of their semi-immune status and because early diagnosis is usually made on the basis of travel history (4). We describe an unprecedented cluster of malaria cases in immigrants from China. Most of the immigrants originated from the province of Zhe Jiang, which is free from indigenous falciparum malaria (Allan Schapira, pers. commun.); therefore, they had no immunity to the infection.

## Case Reports

During the summer of 2000, several Chinese immigrants were admitted to hospitals in northern and central Italy with a diagnosis of malaria. Twenty-two such cases were observed by the Study Group for International Health of the Lombardy Region clinical network (SIRL) and are described here. Diagnoses of malaria and definitions of species were based on microscopic examination of peripheral blood smears. Twenty cases were due to *Plasmodium falciparum*, one to mixed (*P. falciparum*/*P. ovale*), and one to *P. vivax* infection.

The major epidemiologic and clinical characteristics of the 22 cases are listed (Table). All but one were in immigrants arriving for the first time in Italy. A detailed travel history was obtained from half the patients. Communication was extremely difficult, as none could speak any European language or Arabic, and none was accompanied by family members. Even when translators were used, information was incomplete and incoherent, because of fear of deportation. In most cases the area of origin was the province of Zhe Jiang. These patients reported that at least 3 months had lapsed between their departure from China and arrival in Italy. Traveling by land and sea, these immigrants visited several malarious areas of Southeast Asia and eastern and western Africa. Long stops of several weeks in Laos, Thailand, Myanmar, Bangladesh, Pakistan, Kenya, Tanzania, Ivory Coast, or Cameroon were reported. In at least two cases, Italy was not the country of arrival in Europe: one patient entered France first and another entered Switzerland before being transferred to Italy.

Most patients were young adult men. None reported having taken malaria chemoprophylaxis or adopting other malaria preventive measures. Some had had previous febrile episodes, which had occurred outside Italy and had been treated with unspecified drugs. It was almost invariably impossible to define the lag between onset of current symptoms and the time patients sought care at hospital. The time to diagnosis in hospital was longer than 24 hours in 50% of the cases, and it was longer for the first cases occurring at each hospital.

The clinical picture on admission to hospital was that of a nonspecific febrile illness. The parasite density was >10% in 8 (38.1%) of 21 falciparum malaria cases. In 7 (33.3%) of 21 falciparum cases, the criteria for complicated and severe disease (5) were met. One patient died, and another had neurologic sequelae after recovering from cerebral malaria. Patient 6 was admitted to hospital for headache and fever. His condition deteriorated in 24 hours, with the development of coma and hyperbilirubinemia. He was transferred to an intensive care unit 36 hours after admission. Malaria diagnosis was made 48 hours after admission and specific treatment immediately started. He died on the fourth hospital day from multi-organ failure. The autopsy confirmed massive malaria infection in the brain, lungs, heart, kidneys, spleen, and intestine. Patient 15 had a diagnosis of cerebral malaria (convulsions, impaired consciousness), acute renal

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Table. Epidemiologic and clinical characteristics of 22 Chinese immigrants with malaria in Italy

| No. | Sex, age (yrs) | Origin | Countries visited | Day/ mo of initial visit | Malaria species | Parasite density | Days until hospital dx[a] | Clinical signs & symptoms | Treatment | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M, 24 | Na | Pakistan | 1/7 | Pf | <1% | 0 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 2 | F, 20 | Na | Na | 3/7 | Pf | 15% | 0 | Uncomplicated | Mefloquine | Resolved |
| 3 | M, 26 | Na | Na | 3/7 | Pf | 15% | 0 | Uncomplicated | Mefloquine | Resolved |
| 4 | M, 20 | Zhe Jiang | Laos, Myanmar, Bangladesh, Pakistan, East Africa | 5/7 | Pf | <1% | 2 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 5 | M, 29 | Southern China | Pakistan, Myanmar, Thailand | 5/7 | Pf | <1% | 1 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 6 | M, 23 | Na | Vietnam, Cambodia, Kenya | 17/7 | Pf | 20% | 2 | Severe | Quinine + Doxycycline | Death |
| 7 | M, 19 | Zhe Jiang | Pakistan, Ivory Coast | 19/7 | Pf | 3% | 2 | Severe | Quinine + antifolics; transfusion | Resolved |
| 8 | M, 33 | Na | Pakistan | 20/7 | Pv | Nd[2] | 0 | Nd[3] | Chloroquine | Resolved |
| 9 | M, 23 | Na | Na | 20/7 | Pf | 2.5% | 0 | Uncomplicated | Mefloquine | Resolved |
| 10 | F, ? | Na | Pakistan | 21/7 | Pf | 20% | 3 | Severe | Quinine + Doxycycline Exchange transfusion | Resolved |
| 11 | M, 26 | Na | Na | 22/7 | Pf | <1% | 0 | Uncomplicated | Quinine | Self discharged |
| 12 | M, 28 | Na | Tanzania | 22/7 | Pf | 11% | 4 | Severe | Quinine + Clindamicin | Resolved |
| 13 | F, 28 | Na | Na | 26/7 | Pf + Po | 10% | 3 | Severe | Quinine + Doxycycline Primaquine | Resolved |
| 14 | M, 24 | Zhe Jiang | Laos, Myanmar, Bangladesh, Pakistan, East Africa | 27/7 | Pf | <1% | 0 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 15 | M, 25 | Zhe Jiang | Laos, Myanmar, Bangladesh, Pakistan, East Africa | 28/7 | Pf | 15% | 8 | Severe | Quinine + Doxycycline Exchange transfusion | Neurologic sequelae |
| 16 | M, 22 | Zhe Jiang | East Africa | 28/7 | Pf | <1% | 0 | Uncomplicated | Quinine | Resolved |
| 17 | M, 18 | Na | Myanmar, Bangladesh, Pakistan, Ivory Coast, France | 2/8 | Pf | <1% | 1 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 18 | F, 19 | Southern China | Kenya | 6/8 | Pf | 1% | 11 | Uncomplicated | Quinine + Doxycycline | Resolved |

[a]A value of 0 indicates diagnosis on the day of admission.

F = female; M = male; dx = diagnosis; mo = month; Na = not assessed; Nd = not determined in a case of *Plasmodium vivax* infection; Pv = *P. vivax*; Pf = *P. falciparum*; Po = *P. ovale*.

Table, continued. Epidemiologic and clinical characteristics of 22 Chinese immigrants with malaria in Italy

| No. | Sex, age (yrs) | Origin | Countries visited | Day/ mo of initial visit | Malaria species | Parasite density | Days until hospital dx[a] | Clinical signs & symptoms | Treatment | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 19 | M, 29 | Na | Pakistan | 7/8 | Pf | 1% | 0 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 20 | F, 24 | Zhe Jiang | Ivory Coast | 6/9 | Pf | 17% | 2 | Severe | Quinine + Doxycycline Exchange transfusion | Resolved |
| 21 | M, 29 | Southern China | Pakistan, Ivory Coast | 11/9 | Pf | <1% | 0 | Uncomplicated | Quinine + Doxycycline | Resolved |
| 22 | M, 18 | Na | Cameroon | 15/11 | Pf | <1% | 0 | Uncomplicated | Mefloquine | Resolved |

[a]A value of 0 indicates diagnosis on the day of admission.

F = female; M = male; dx = diagnosis; mo = month; Na = not assessed; Nd = not determined in a case of *Plasmodium vivax* infection; Pv = *P. vivax*; Pf = *P. falciparum*; Po = *P. ovale*.

failure, and adult respiratory distress syndrom. He received intravenous quinine, doxycycline, and exchange transfusion, and was discharged in apparently good health 24 days after admission. He was readmitted 1 week later with a neurologic syndrome that required a 7-day hospital stay. The final diagnosis was acute psychosis, which was considered a consequence of the previous episode of cerebral malaria. Patients 10 and 20 had cerebral malaria. They were both treated with intravenous quinine, doxycycline, and exchange transfusion and recovered. Patient 12 also had cerebral malaria, was treated with intravenous quinine and clindamicin, and recovered. Patient 7 had acute anemia and received a blood transfusion in addition to quinine and doxycycline. Uncomplicated cases received either quinine or mefloquine, and all recovered. Patient 11 left the hospital on the same day of admission, without completing his malaria treatment course.

## Conclusions

Malaria is a reemerging problem in Italy, with more then 5,000 cases reported from 1986 to 1996 (6). Most cases are imported and present as sporadic disease. The only major cluster of malaria, reported by Orlando and colleagues, was caused by the practice of sharing injection equipment among drug addicts (7). We describe an unprecedented cluster of imported malaria in Chinese clandestine immigrants. Although Italy is a natural point of arrival for immigrants to Europe, it is unlikely that the cluster was limited to Italy: at least two of our patients visited other European countries before entering Italy.

This cluster deserves some discussion. First, malaria prevention in travelers requires active medical interventions and personal motivation for both chemoprophylaxis and prevention of contact with the vector (8). Clandestine migrants are unlikely to receive such medical attention, and none of our patients reported having taken malaria prophylaxis. Second, falciparum malaria in nonimmune persons is potentially fatal and thus needs to be diagnosed and treated early. A diagnostic delay almost certainly occurred among these immigrants, mainly because clandestine immigrants are unlikely to seek prompt medical attention. Although this could not be confirmed, the high parasite density at admission to hospital is an indirect indicator. After hospital admission, diagnostic delay was higher for the first cases and decreased thereafter: in the absence of an alert system each hospital had to learn for itself about the possibility of malaria in Chinese immigrants. Third, travelers from nonmalarious areas pose a difficult diagnostic dilemma to health-care providers, who require a detailed travel history to raise the suspicion of malaria. Language barriers and fear of deportation made this step very difficult in our case list. Even when translators were used, patients were still very reluctant to provide the details of their trip to Italy because of their illegal status. It is also possible that they indeed did not know details about the trip.

The clinical consequence of the factors mentioned above was that at hospital admission cases were more severe than average. The rate of severe malaria in the cluster was 33%, which is much higher than the rates of 1.3% in immigrants and 9.2% in nonimmune Italians recently reported for Lombardy region (9). One patient died, and another one had acute psychosis as a consequence of cerebral malaria.

Italy, from which malaria was eradicated in the 1950s, is susceptible to the reintroduction of the disease because the vector, mainly *Anopheles labranchiae*, is still present at high densities (10). *P. vivax* malaria transmitted by indigenous vectors has recently been documented in Maremma, Italy (11). Italian vectors have been found to be refractory to infection by strains of *P. falciparum* from tropical Africa in in-vitro studies (12). However, the risk of reintroduction of *P. falciparum* strains from Asia, where local vectors are more closely related to the Mediterranean ones, is basically unknown.

Malaria in Chinese immigrants may represent an emerging problem in Europe, one which might increase the rate of severe disease and death from imported falciparum malaria. Interventions to raise awareness of the malaria risk among the Chinese community of clandestine immigrants may not be feasible. To limit the risk for severe disease and death, local health structures in the countries of arrival need

to be alert to the possible risk of malaria in febrile illegal immigrants even if they are not coming from classical high-risk areas. This cluster was reported to TropNetEurop, a network which has sentinel clinics throughout Europe, and GeoSentinel, a similar network mainly made up of centers in the United States. A local reporting system could have helped in this specific situation to quickly generate awareness of the outbreak.

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# Three Cases of Bacteremia Caused by *Vibrio cholerae* O1 in Blantyre, Malawi

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We report three fatal cases of bacteremia (two adults, one neonate) caused by *Vibrio cholerae* O1 (Ogawa), which occurred in the context of a community outbreak of cholera diarrhea in Blantyre, Malawi. Only four cases of invasive disease caused by *V. cholerae* O1 have previously been reported. We describe the clinical features associated with these rare cases and discuss their significance.

*Vibrio cholerae* O1 and O139, the causative agents of cholera, are morphologically and biochemically identical to the other non-O1 *V. cholerae*, but antigenically, epidemiologically, and clinically distinct. Non-O1 *V. cholerae* can cause small outbreaks of diarrheal illness related to contaminated seafood. There are, however, numerous case reports of bacteremia caused by non-O1 *V. cholerae* in persons with predisposing conditions, most commonly cirrhosis (1) but also nephrotic syndrome, diabetes, hematologic malignancy, gastrectomy, and AIDS/lymphoma (2).

*V. cholerae* O1 and O139, by contrast, cause epidemic diarrheal disease. *V. cholerae* O1, in particular, is reputed to be noninvasive. Only three cases of bacteremia and one case of meningitis caused by *V. cholerae* O1 have been reported, from Australia (3), southern Africa (4), Pakistan (5), and Mexico (6) (Table). We report a series of three cases of bacteremia caused by *V. cholerae* O1 from a single center in sub-Saharan Africa (Queen Elizabeth Central Hospital [QECH], Blantyre, Malawi), which occurred in the context of a community outbreak of cholera.

### Cholera Outbreak

The three cases of bacteremia occurred during and after a cholera outbreak in Blantyre, Malawi, during March 1998, in which 178 adults (ages 15 to 68 years), 64 children (aged 1 month to 14 years), and 2 neonates were admitted to QECH with cholera diarrhea. Case 1 (neonate) occurred during the outbreak in March, and Cases 2 and 3 (adults) were among the sporadic cases at QECH during the following 12 months.

The first cases in the outbreak were identified by stool culture; thereafter, stool cultures were systematically obtained for 1 in 10 of suspected cases, to monitor the outbreak. Median intravenous fluid requirement for adult cases was 11 L (range 2 to 36). A single dose of doxycycline was prescribed for all suspected cases. There were two adult and two pediatric deaths during the March outbreak (overall death rate 1.6%), including Case 1 with cholera bacteremia. The three deaths not described below were attributed to acute severe dehydration, and one was associated with second-trimester abortion. During March 1998, adult patients were admitted and nursed adjacent to the wards in a cholera tent, where blood cultures were not routinely performed. After March 1998, sporadic cases (including Cases 2 and 3) continued to come to QECH; these patients were admitted to the general medical wards of the hospital. Blood cultures were routinely obtained for patients with fever and shock; such patients were cared for in the diarrhea bay of the medical wards.

## Case Reports

### Case 1 (Neonate)

A male twin was born in QECH in March 1998, at 34 weeks' gestation, by spontaneous vaginal delivery; he was breastfed. He was well until day 2, when he became hypothermic, hypoglycemic, and peripherally cyanosed. He had no diarrhea. Blood culture was taken, treatment with penicillin and gentamicin was begun, and expressed breast milk was fed by nasogastric tube, but the child died 13 hours later. Blood culture grew *V. cholerae* O1 at 24 hours (cloudy bottle). A stool culture was not taken.

The second twin followed a similar clinical course and died on day 2. Blood culture was negative. The mother was a healthy 21-year-old, with no diarrheal disease. We were unable to recall her for stool culture.

### Case 2 (Adult)

A previously healthy 45-year-old woman was admitted to QECH in September 1998 with profuse, watery diarrhea. She was afebrile, dehydrated, and tachycardic with thready pulses, and was managed with 11 L of intravenous Ringer's lactate followed by oral rehydration therapy (ORT). Her diarrhea became bloody, blood culture was taken, and nalidixic acid was given empirically. Over 36 hours her diarrhea resolved, her clinical state improved, and she was able to move around, but she died suddenly on day 4 after an

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Table. All reported cases of invasive disease caused by *Vibrio cholerae* O1, in chronological order

| Age, sex | Susceptibility | Clinical features | Outcome | Ref |
|---|---|---|---|---|
| 6 years, female | Autoimmune disease, achlorhydria | Diarrhea, severe sepsis syndrome | Survived after intensive therapy | 3 |
| 6 days, male | Neonate | Diarrhea, afebrile, neutrophilia, uremia | Died | 4 |
| 8 months, female | None | Diarrhea, febrile, neutrophilia | Survived with rehydration and antibiotics | 5 |
| 6 years, female | Chemotherapy | Meningitis, blood culture negative | Died | 6 |
| 2 days, male | Neonate | No diarrhea | Died | TR |
| 45 years, female | None | Diarrhea transiently bloody, afebrile | Died | TR |
| 65 years, female | None | Diarrhea, neutrophilia, renal failure secondary to dehydration | Died of renal failure after 2-3 weeks | TR |

TR = this report; see text.

unwitnessed collapse. *V. cholerae* O1 was grown at 24 hours (cloudy bottle). Stool culture had not been taken.

### Case 3 (Adult)

A previously healthy 65-year-old woman initially visited an outlying rural health center in February 1999 with sudden onset of profuse watery diarrhea. She was treated with 35 L of intravenous fluid followed by ORT for 4 days. She was not given antibiotics, her diarrhea ceased, and she was discharged. The water supply in her village was a covered well, and there was one simultaneous case of cholera diarrhea in the area, in a young woman, who fully recovered.

Over the next 3 days, Patient 3 had anuria, confusion, and shivering but no further diarrhea. She was taken to QECH, and on admission was afebrile, in shock, dehydrated, and confused. A clinical assessment of dehydration and sepsis prompted empiric management with intravenous rehydration, chloramphenicol, and gentamicin.

Blood tests revealed a leukocyte count of 22 x $10^9$/L (88% neutrophils), $Na^+$ 173 mmol/L (normal 135-145), $K^+$ 3.8 mmol/L (normal 3.5-5.0), and urea 71 mg/dL (normal 8-25). Liver function tests, urine examination, and chest X ray were normal. *V. cholerae* O1 was grown from blood at 36-48 hours (routine subculture) and was found to be sensitive to erythromycin but resistant to ampicillin, chloramphenicol, cotrimoxazole, and tetracycline; antibiotic therapy was changed accordingly. Blood culture taken after 7 days of treatment with erythromycin was negative. Rectal swab and urine cultures were negative. HIV serologic testing was negative. Despite rehydration and good subsequent urine output, she remained in renal failure with presumed acute tubular necrosis secondary to inadequate rehydration during her original diarrheal illness. She died 14 days after admission.

### Genomic Analysis

For adults, 5 mL of venous blood was incubated in a single aerobic culture bottle of 50 mL brain heart infusion broth containing sodium polyanetholesulphonate (E&O Laboratories, United Kingdom) at 37°C in air. For neonates, 2 mL of blood was incubated in 20 mL of broth in the same manner. Routine blinded subcultures on sheep blood agar incubated in $CO_2$ were performed at 24 and 48 hours and at 7 days. Bottles appearing cloudy were examined by Gram stain and then subcultured onto appropriate media, dependent on Gram stain findings. Antibiotic susceptibility testing was performed by disk diffusion. The organisms were identified biochemically and serologically as *V. cholerae* O1 (Ogawa).

Blood culture isolates from Cases 1 and 3 were available for subsequent genomic analysis. 16S rRNA sequence analysis was performed by using universal oligonucleotide primers (7). The 1,500-bp product was extracted from the gel and sequenced on an ABI PRISM system (Applied Biosystems, Perkin Elmer Corp, Foster City, CA). The 16S sequence was submitted to GenBank-BLAST Search for analysis. Multiplex polymerase chain reaction (PCR) was used to determine the presence of important virulence factors, namely, cholera toxin (*ctx*), toxin-regulated pilus (*tcp*), and the global regulatory element *toxR*, as described (8). Plasmids were extracted from control (*Escherichia coli* 39R861, *E. coli* V517) and test bacteria (Plasmid mini kit, Quiagen Ltd., Germany) and separated by electrophoresis. Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA following digestion with the restriction endonuclease *Spe1* was performed. Clonal relatedness of the cholera isolates was assessed according to the criteria of Tenover (9).

16S rRNA sequence analysis confirmed both isolates as *V. cholerae*. Multiplex PCR amplicons of the appropriate size were detected for *ctxA* (301 bp), *tcpA* (618 bp), and *toxR* (900 bp). No plasmids were detected from the test isolates (the upper limit of plasmid size detection was 160 kbp). The two isolates were indistinguishable by PFGE of macrorestricted chromosomal DNA.

## Conclusions

This is the first reported series of *V. cholerae* O1 bacteremic cases. Biochemical, serologic, and genomic analysis confirmed the identity of the organisms as *V. cholerae* O1 (Ogawa).

These isolates could have been contaminants, arising on the ward or in the laboratory. Several features, however, make this unlikely. Skin was disinfected before blood was taken from the antecubital fossa, and a pure growth without skin contaminants was obtained in all three cases after 24 to 48 hours. Cases 2 and 3 postdated the main cholera outbreak, so the patients were not in a cholera tent and samples were not taken in an epidemic situation. There were no other coincident cases of cholera on the ward at the time. Moreover, in Case 3 a rectal swab culture was negative at patient

admission, and the blood culture sample was taken in the general medical admissions area before the patient was transferred to the diarrhea bay. The blood culture specimens were handled in a research laboratory, in a separate building from the government laboratory where all stool cultures were performed. The three isolates could not be linked to any single technician or ward nurse, nor were they clustered in time. Finally, the high case death rate compared with the 1.6% overall death rate suggests that the isolates were of clinical relevance. Previously reported cases also show a poor outcome (Table).

Why did we observe bacteremia? All the cases we describe had unusual features or complications. Case 1 had no diarrheal illness, Case 2 had transient bloody diarrhea, and Case 3 was in an elderly woman who had renal failure secondary to inadequate initial rehydration. Invasive *V.cholerae* O1 disease has been associated with autoimmune disease, achlorhydria, and chemotherapy in two of the four previously reported cases (3,6), but our adult patients did not have known longstanding immunosuppression. HIV disease is common in Blantyre and is associated with bacteremia caused by *Streptococcus pneumoniae* and nontyphoid salmonellae (10), but no reports link HIV with severe or invasive *V. cholerae* O1 infections. *V. cholerae* O1 was grown from the stool of 5 of 77 Guatemalan AIDS patients; none had a fatal outcome, and 4 had only mild diarrhea. Three of these cases had enteric coinfection with *Cryptosporidium* or nontyphoid *Salmonella* (11).

Case 2 had transient bloody diarrhea, unlikely to be caused by *V. cholerae* alone. It is noteworthy that *V. cholerae* (unknown serogroup) and *Salmonella enterica* serotype Typhi were simultaneously isolated from blood in a 1932 case (12). Enteric bacterial coinfection may have facilitated mucosal invasion by *V. cholerae* in both these cases.

Cholera is well described in children <2 years of age, and breast feeding is protective (13). Cholera diarrhea is, however, extremely rare in neonates. (We found two cases with positive stool cultures during this outbreak.) Colostrum may offer potent protection among breastfed neonates in disease-endemic areas, mediated by specific immunoglobulin (Ig) A (14). Despite breastfeeding, however, Case 1 may have acquired *V. cholerae* O1 infection during birth from a mother with asymptomatic stool carriage (common during an outbreak). The early events of infection or invasion could have occurred before the first colostrum feed; the onset of symptoms on day 2 of life would be in keeping with this. The previously reported neonatal case (4) also had a healthy mother and onset of symptoms on day 5 of life.

The true incidence of bacteremia during this outbreak is unknown, as blood cultures were not routinely taken in the cholera tents. While *V. cholerae* O1 bacteremia is apparently a rare event, reported cases suggest that persons at risk include those with underlying immunosuppression (chemotherapy, autoimmune disease, achlorhydria), the elderly, and neonates. Enteric bacterial coinfection may play a role in invasion. There is no evidence that HIV infection is a risk factor. Intravenous rehydration and ORT remain the mainstays of successful treatment, but our experience reemphasizes the importance of antibiotics as adjunctive treatment.

What could be the route of invasion of *V. cholerae* O1? Intestinal M cells are enterocytes adapted to sample enteric organisms, which are then translocated to gut lymphoid tissue, where a specific sIgA response is generated. Viable *V.cholerae* O1 organisms are translocated across the mucosa in this manner by M cells. This has been proposed as the route by which *V. cholerae* O1 may in some circumstances cause bacteremic illness (15).

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# Rabies in Marmosets (*Callithrix jacchus*), Ceará, Brazil

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A new *Rabies virus* variant, with no close antigenic or genetic relationship to any known rabies variants found in bats or terrestrial mammals in the Americas, was identified in association with human rabies cases reported from the state of Ceará, Brazil, from 1991 to 1998. The marmoset, *Callithrix jacchus jacchus* , was determined to be the source of exposure.

Canine rabies has been controlled in southern Brazil, and cases have declined in the rest of the country (1). Under these new epidemiologic conditions, the existence of rabies in other species, until now eclipsed by the rabies cycle in dogs, has become evident (2-4).

In the state of Ceará, for example, 13 human deaths due to rabies transmitted by wildlife were reported from 1991 to 1998 (Figure 1). Surveillance data indicated that these deaths were the consequence of exposure to bats (four deaths), a crab-eating raccoon (*Procyon cancrivorous* [guaximín]; one death), and the white-tufted-ear marmoset (*Callithrix jacchus jacchus* [sagüí]; eight deaths). These last eight cases constitute the first report in which one species of the order Primate is a primary source of rabies infection for humans in a restricted geographic area.

The marmoset, *C. j. jacchus* or sagüí, is a small diurnal primate that feeds on insects, fruits, and tree exudates (5). Members of this species are commonly captured to keep as pets in Ceará, as other marmosets are in the rest of the country (6) (Figure 2). The sagüí is also present in the neighboring states of Piauí and Pernambuco (Figure 1) (5). These marmosets are highly adaptable to different habitats and can be found on plantations and in urban parks (5,7).

In one of the eight human rabies cases associated with *C. jacchus*, the animal approached the house and attacked the owner. In another case, the marmoset was raised as a pet. In the other cases, the exposure occurred during attempts to capture the animals (4). Seven of these cases occurred in the coastal region, where the sagüí is more abundant (4). Epidemiologic investigations and surveillance data suggested the emergence of a rabies cycle in which this marmoset was the main transmitter. The public health importance of this situation is reflected not only in reported human deaths, but also in the fact that in Ceará an average of 25 persons per month seek rabies postexposure prophylaxis for marmoset and other primate bites (4).

To better understand the underlying factors that could be responsible for the emergence of these unusual epidemiologic events and to help identify a possible reservoir of this *Rabies virus* (RABV) circulating in Ceará, three rabies field isolates—two obtained from humans, each bitten by a different sagüí (Brhm4097 and Brhm4108), and one from a rabid sagüí (Brsg4138) in 1998—were antigenically and genetically characterized.

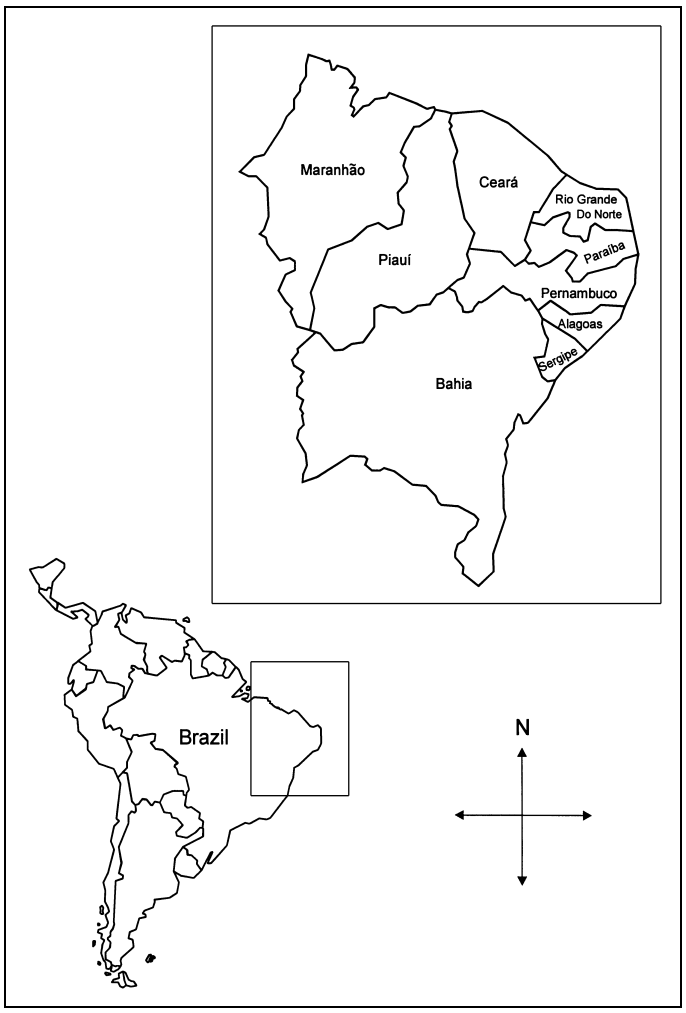


Figure 1. Map of Brazil indicating the location of Ceará State.

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Figure 2. A pet marmoset with its owner.

## The Study

Case Brhm4097 was in a 31-year-old woman who died with a clinical diagnosis of rabies, later confirmed by a direct immunofluorescence-antibody (DIF) test. Interviews with the patient and her relatives indicated that she had been attacked and bitten on the leg by a sagüí near her house. Case Brhm4108 was in a 17-year-old woman who was hospitalized with a presumptive case of meningitis. Upon evaluation, a clinical diagnosis of rabies was made and later confirmed with a DIF test. Interviews with the patient and family members revealed that she had been bitten on one ear by a sagüí captured in the forest 4 days earlier. In both cases, those bitten did not request postexposure prophylaxis because they were not aware of any potential risk for rabies transmission by this animal. Case Brsg4138 was a *C. j. jacchus* kept as a pet, which was brought to health authorities because it exhibited behavioral changes. This marmoset was euthanized and sent to the laboratory to be tested for rabies; RABV antigen was identified in its brain with a DIF test.

These three isolates were compared with RABV variants circulating among dogs in Latin America, with other RABV variants maintained by sylvatic terrestrial reservoirs in the United States (north-central skunk, south-central skunk [*Mephitis mephitis*], Arizona and Texas fox [*Urocyon cinereoargenteus*], California skunk [*M. mephitis*], Texas coyote [*Canis latrans*], and raccoon [*Procyon lotor*]) and with isolates obtained from terrestrial wildlife in Latin America in which the reservoirs were either unknown, e.g., a fox in Peru and a cat in Venezuela, or unconfirmed, e.g., skunks in Mexico. The three Brazilian viruses were further analyzed and compared with RABV from endemic cycles maintained in migratory bats (red bat [*Lasiurus cinereus]*, free-tailed bats [*Tadarida brasiliensis*], nonmigratory insectivorous bats (big brown bat [*Eptesicus fuscus],* and California myotis [*Myotis californicus*]) in the United States. South American RABV isolates obtained from vampire bats (*Desmodus rotundus*), vampire bat-related cases, and insectivorous bat species that are known rabies reservoirs (lasiurine species and free-tailed bats) or unconfirmed reservoirs were also included in this comparison (8-12).

Antigenic characterization was conducted with a panel of eight monoclonal antibodies (MAbs) elicited against the viral nucleoprotein (8). Primary isolations were made by mouse intracranial inoculation. Acetone-fixed touch impressions of infected mouse brain material were reacted with the MAbs by indirect immunofluorescence techniques (13,14).

Antigenic characterization of the three isolates revealed only one reaction pattern. The isolates reacted positively with MAb C9 and C10 and negatively with the other six MAbs. Comparison of this reaction pattern with those that characterize RABV variants in known reservoirs in the Americas (13,14) indicated that the antigenic profile of these three viruses has not been previously identified.

Genetic analysis of the samples was performed by sequencing part of the nucleoprotein gene (from position 1,157 to position 1,476, as compared with RABV SADB19 [15]), then comparing its phylogenetic relationship with the RABV variants described above (8,12).

For the sequencing studies, viral RNA was extracted from the infected tissues with TRIzol (Invitrogen, San Diego, CA). The cDNA was obtained by reverse transcription-polymerase chain reaction techniques by using primers 10g and 304, as described (8,16). The cDNA was sequenced with primer 304 by using the Taq Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA) on an Applied Biosystems 377 DNA automated sequencer (Applied Biosystems), as reported (8).

For phylogenetic analyses, the PileUp program of the Wisconsin Package Version 10.1 (Genetic Computer Group, Madison, WI) (17) was used to construct the alignment of viral sequences. Further analyses were carried out with distance matrix methods, as implemented in the PHYLIP Package, Version 3.5 (18), using the programs DNADIST (Kimura-two parameter method) and NEIGHBOR (neighbor-joining method). The confidence value for each node (100 replicates) was assessed with the SEQBOOT program, and the consensus tree was obtained with the CONSENSE program of the same package. Trees were constructed with the TREEVIEW program (19).

Comparative phylogenetic analyses by the distance matrix methods yielded a tree in which all viruses segregated into two main groups, identified as A (bootstrap value 57%) and B (bootstrap value 99%) (Figure 3). The Ceará viruses were segregated in group A, which included RABV genetic variants circulating in hematophagous and nonhematophagous bats in the Americas and viruses of raccoon and south-central skunk cycles in the United States. The topologic association between RABV genetic variants circulating in well-recognized terrestrial reservoirs and variants maintained by bat species has been previously described (20). Group B was formed by viruses circulating in dogs and terrestrial wildlife in the Americas.

In group A the Brazilian isolates clustered together, forming a discrete clade A1 (bootstrap 100%) demonstrating their distinct nature (Figure 3). Viruses Brsg4108 and

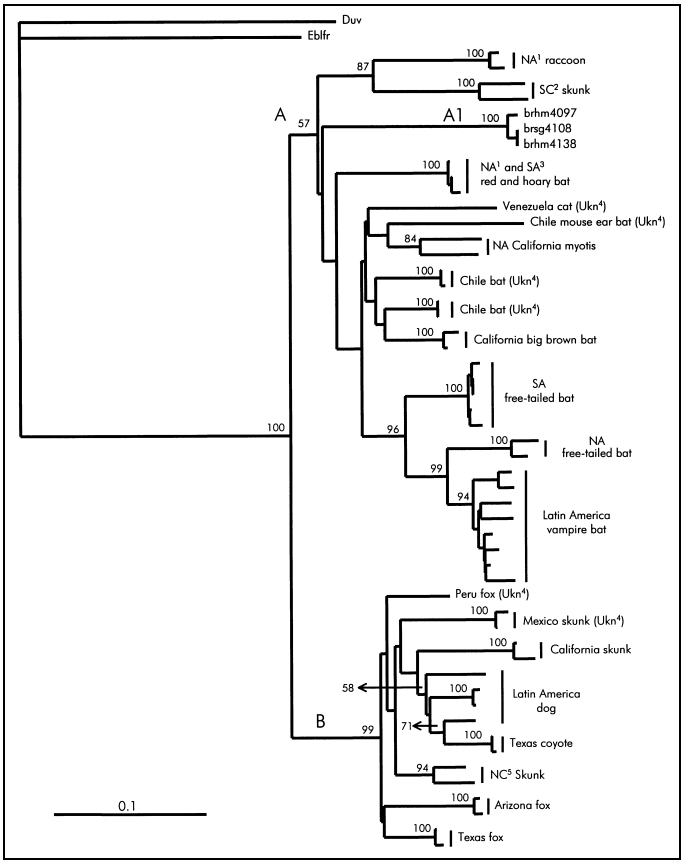


Figure 3. Neighbor-joining tree of the comparison of the Ceará samples with isolates obtained from domestic and wild animals from the Americas. Bootstrap values obtained from 100 resamplings of the data using distance matrix methods are shown at the corresponding nodes. Only bootstrap values >50% are shown at branching points. Bar in left corner indicates 0.1 nucleotide substitutions per site. Significance of letter designations at the nodes are discussed in the text. a: North America; b: south central USA; c: South America; d: unknown reservoir; e: north central USA.

Brhm4138 showed no nucleotide difference between them and revealed a genetic distance value of only 1.3% with sample Brhm4097. The remarkable genetic relatedness of the group A1 members was in clear contrast to the high percentage of genetic distance that they showed with all other rabies variants. The genetic distances between Group A1 and all other rabies variants ranged from 20% (lasiurine species) to 34% (skunks from Mexico). Although bootstrap analyses highly supported terminal nodes that clearly defined each of the rabies genetic variants in group A, as in group B this method did not render significant values in most of the intermediate nodes; thus, no genetic relationships between the Ceará viruses and the other variants could be determined.

## Conclusions

The high degree of genetic homology among the Ceará rabies viruses, in conjunction with surveillance data, demonstrated an epidemiologic linkage between the viruses and confirmed that they represent a unique and independent rabies endemic cycle.

These findings are also noteworthy because nonhuman primates have rarely been reported rabid in the wild, and they have been involved only sporadically in cases of human exposures to RABV (21-23). Rabies affects wild and domestic animals as well as humans. Minor changes in any of these populations or in their environment can result in the emergence or reemergence of the disease in a geographic area (24). Several probable factors could have caused the emergence of rabies in the *C. j. jacchus* population of Ceará. First, small changes produced by human activities are not necessarily deleterious for survival of *C. j. jacchus*. Indeed, such modifications might be beneficial for this species in the long term and could contribute to an increase in its population (6). The proximity of this marmoset to urban settlements and the common practice of capturing and keeping them as pets are two other contributing factors. Additionally, both the reduction in rabies incidence among dogs in the state (1,2) and the improvement of rabies surveillance programs by incorporating the molecular characterization of rabies isolates contributed to the recognition of this cycle in Ceará.

Wildlife unable to support rabies endemic cycles are sporadically infected (spillover) as a result of their interaction with rabid animals from sympatric species capable of perpetuating the virus in nature (reservoirs). The infection of *C. j. jacchus* with this distinct RABV genetic variant could be the result of spillover from an unknown reservoir. Until now, available data did not provide enough evidence to determine the nature (Chiroptera vs. Carnivora) of this putative reservoir. Although mostly arboreal, *C. j. jacchus* descends to the ground to feed on insects or to cross clearings in the forest, behaviors that may expose them to bats or terrestrial reservoirs. Alternatively, these events could be evidence of *C. j. jacchus* acting as a rabies reservoir. To act as a rabies reservoir, a species should be highly susceptible to the virus, undergo variable incubation periods, excrete the virus in saliva at the appropriate concentration to infect conspecifics, and maintain a high population density and turnover (25).

Field studies of *Callithrix* spp. are limited (7), but some characteristics of the natural history of this species correspond to those of a typical rabies reservoir. Callitrichids are some of the most successful species in regard to geographic distribution, population density, and habitat exploitation (26). A population of 700 animals per square kilometer was observed in a 3-Ha forest fragment in Brazil (27). Although as yet no experimental inoculation studies mimicking the natural route of infection have been conducted to investigate the pathobiology of rabies in *C. j. jacchus*, this animal is highly susceptible to the intracerebral inoculation of the virus (28). Improvement in basic surveillance programs for rabies in wildlife, public education, and determination of rabies seroprevalence in *C. j. jacchus* in Ceará would help elucidate this new epidemiologic puzzle.

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# *Aedes* (*Stegomyia*) *albopictus* (Skuse), a Potential New Dengue Vector in Southern Cameroon

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*Aedes albopictus*, a mosquito vector of *Dengue virus* , has been recorded for the first time in Cameroon. Entomologic surveys in 2000 demonstrated that it is widespread in southern Cameroon, colonizing a wide variety of breeding sites and biting humans in every district surveyed. The presence of this vector increases the risk for emergence of dengue in Cameroon.

*Aedes albopictus* is among the most important arbovirus vectors in the world, particularly for *Dengue virus* (DV) (1). The microhabitats of its larvae are mainly tree holes and a wide variety of containers. The eggs can survive desiccation for several months. The adult biology of *Ae. albopictus* is similar to that of the urban population of *Ae. aegypti*, a dengue and yellow fever vector (2). The characteristics of its eggs, its close association with humans, and increasing intercontinental travel have favored the expanding global distribution of this Asian species (3).

*Ae. albopictus* was recorded in North America as early as 1972. Established populations were detected in 1985, imported from Asia in used tires (4). Its presence was reported in Brazil in 1986, then in the Pacific islands and the Caribbean islands, and more recently in Europe (Albania, Italy, and France) (5,6).

In Africa, this vector was observed for the first time in 1989 in South Africa. After its eggs were introduced in tires from Japan (7), *Ae. albopictus* was recorded in Nigeria in 1991 (8), where it has become widespread. To date, this vector has not been observed in other sub-Saharan countries.

Surveys of *Ae. aegypti* distribution conducted from 1950 to 1995 in several regions in Cameroon did not record *Ae.albopictus*. A large trial conducted in 1976 in 84 locations recorded 1,112 *Ae. aegypti*-positive larval development sites but none positive for *Ae. albopictus* (9). Moreover, entomologic investigations during two yellow fever epidemics in 1990 and 1995 in North Cameroon recorded only *Ae. aegypti* (10; unpub. report: Enquête entomo-épidémiologique sur deux cas mortels de fièvre jaune survenus dans la ville de Ngaoundéré [Province de l'Adamaoua, Cameroun], ORSTOM laboratory, Centre Pasteur of Cameroon, 1995). DV has never been isolated in Cameroon.

In October 1999, one of the authors captured biting *Ae.albopictus* females, which prompted a thorough investigation to monitor the presence, distribution, and biology of this species in southern Cameroon.

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## Materials and Methods

### Study Sites

Surveys were conducted in the two main cities of Cameroon: Douala, pop. 1,400,000 (4°00'N, 9°45'E), commercial harbor and largest city in Cameroon, and Yaoundé, pop. 1,300,000 (3°4150'N, 11°30'E) the capital city, located at an altitude of 800 m. Entomologic studies were also conducted in Campo (2°30'N, 9°50'E; pop. 4,000), Edea (3°45'N, 10°10'E; pop. 100,000), and Bafia (4°45'N, 11°15'E; pop. 50,000).

### Larvae and Adult Mosquito Collections

Larval development sites of mosquitoes were investigated in four districts in Yaoundé (Gare, Cité Verte, Brasseries, and Biyemassi), four districts in Douala (Dibom, New Bell, Bonaberi, and Makepe), and three districts in Edea and Bafia. Approximately 20 potential breeding sites containing water were sampled in each district in Yaoundé and Douala; an average of seven breeding sites were sampled in each district in Edea and Bafia. A breeding site was recorded as positive when it contained mosquito larvae or pupae, whatever the species.

Biting behavior of mosquitoes was checked by five adult volunteers in the districts of Yaoundé, Douala, and Campo. These volunteers collected mosquitoes landing on their arms or legs from 5:00 to 6:30 p.m. All surveys were conducted in October and November 2000, at the end of the long rainy season.

Larvae and adults were identified by the morphologic identification keys and morphologic descriptions of African *Aedes* species (11-13). Male genitalia were dissected and examined under a microscope.

## Results

*Ae. albopictus* was present in all five towns and in every district sampled. Species identification was confirmed on larvae and adult males and females. Of the positive larval development sites sampled, 75% of 36 in Yaoundé and 45% of 53 in Douala contained *Ae. albopictus* larvae. *Ae. albopictus* was found in five breeding sites in Edea and seven in Bafia (Table).

The volume of water in *Ae. albopictus*-positive breeding sites ranged from 50 mL to 100 L. Species found together in

Table. Breeding sites found positive for *Aedes albopictus* in 2000, southern Cameroon

| Types of breeding sites containing water | Number of positive / sites sampled of each type | Percent Positive (%) |
|---|---|---|
| Used tire | 36/77 | 47 |
| Plastic container | 7/27 | 26 |
| Can and broken bottle | 9/30 | 30 |
| Plastic cup | 3/6 | 50 |
| 200-L barrel | 0/7 | 0 |
| Abandoned car part | 6/35 | 17 |
| Cement washtub | 0/4 | 0 |
| Flowerpot | 0/2 | 0 |
| Tree hole | 0/4 | 0 |
| Cow horn | 0/4 | 0 |
| Cocoa pod | 0/4 | 0 |
| Enameled plate | 1/6 | 17 |
| Snail shell | 1/3 | 33 |

the same sites were *Ae. aegypti*, *Anopheles gambiae* s.s., *Culex* gr. *decens*, *Cx. quinquefasciatus*, *Cx. poicilipes*, *Cx. duttoni*, *Cx.* (*Culiciomyia*) sp., *Cx.* (*Lutzia*) *tigripes*, and *Eretmapodites quinquevittatus*. Of breeding sites positive for *Ae. albopictus* or *Ae. aegypti*, both species were found together in 68% of sites in Yaoundé, 50% in Douala, 33% in Edea, and 38% in Bafia.

Late afternoon captures of adults demonstrated that *Ae.albopictus* is anthropophilic. The average number of *Ae.albopictus* females collected per volunteer from 5:00 to 6:30 p.m. was 1.1 (range 0 to 8) in Douala and 3.0 (range 0 to 17) in Yaoundé. Other species collected were *Ae. aegypti*, *An. gambiae* s.s., *Cx. quinquefasciatus*, *Cx. antennatus*, *Cx. perfuscus*, *Cx.* from *neavei* group, *Cx.* from *decens* group, *Er. quinquevittatus*, *Mansonia uniformis*, and *Ma. africana*. *Ae. albopictus* was the species most often captured, accounting for 35% of all the mosquitoes.

## Conclusions

In 2000, *Ae. albopictus* was already widespread in South Cameroon. It was present in all the districts and towns sampled, in a wide variety of breeding sites, the most common being used tires, as described elsewhere (2). Used or retread tires are imported regularly from the United States, Nigeria, and South Africa, countries where *Ae. albopictus* is present (unpub. data, Ministry of Commerce, Cameroon). This observation strongly suggests that this species was introduced to Cameroon in this way, likely on multiple occasions in different regions.

The species is frequently associated with *Ae. aegypti*, as observed in other countries (14). Some observations from regions where *Ae. albopictus* was recently introduced suggest it tends to supplant *Ae. aegypti* (15). Such interspecific competition was experimentally observed in an insectary (16). The absence of *Ae. albopictus* in the lists of mosquito species observed in Cameroon before 1995 suggests that this species has colonized South Cameroon recently and that its diffusion has been rapid, as was the case in neighboring Nigeria and in America and Europe.

*Ae. albopictus* is a competent vector for DV. Because this disease is expanding in the world (17), data are needed on the actual distribution of *Ae. albopictus* throughout Cameroon and the potential risk for transmission of arbovirus. Surveillance of used tires, which seem to be its preferred breeding sites, can provide maximum information on species distribution at the lowest cost-effective rate. The presence of this vector, in association with *Ae. aegypti*, increases the risk for emergence of dengue in Cameroon.

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## Usefulness of Seminested Polymerase Chain Reaction for Screening Blood Donors at Risk for Malaria in Spain

**To the Editor:** Assurance of blood safety relies on effective public health surveillance for infectious diseases. Appropriate management of emerging disease threats requires that this surveillance be combined with screening measures to eliminate or minimize the risk of transmitting transfusion-associated disease. Diseases with a long and relatively asymptomatic period (malaria) during which microorganisms are present in the blood are of particular concern for transfusion safety.

Many reports have shown that falciparum malaria can remain in the bloodstream for a long time (even years) as an asymptomatic infection (1). Parasitic contamination of donated blood can occur only if the donor has parasitemia, usually asymptomatic, at the time the blood is collected.

In accordance with legislation of the European Community and the United States, travelers who have visited malaria-endemic areas may be accepted as regular blood donors 6 months after return to the nonendemic area, providing they have been free of unexplained febrile illnesses and have not taken antimalarial drugs (2,3). Immigrants or visitors from endemic areas may donate blood 3 years after they leave the area if they have been asymptomatic in the interim. Donations to be used for the preparation of plasma, plasma components, or derivatives devoid of intact erythrocytes are exempt from these restrictions.

Transfusion-associated cases of malaria have occurred in recent years in the European Community and the United States, although the residual risk of receiving a unit of erythrocyte concentrate contaminated with malaria parasites is very low (estimated at 1 case per 4 million units donated) (4). On the other hand, the potential survival of the organism in an infectious form during blood processing and storage should also be investigated. Several reports concur that all *Plasmodium* species can remain viable in stored blood for at least a week (*P. falciparum* malaria has been transmitted by blood stored for 19 days) (5).

The increase in tourism and migration has caused an increase in imported cases of malaria infections in returned travelers and immigrants from malaria-endemic areas. The number of immigrants from different geographic areas of the world is changing the demographic pattern of European countries, including Spain. Since the early 1990s, the birth rate in the migrant population has attained the same rate as that of the Spanish autochthonous population. This fact has led the migrant population to be incorporated into every layer of society, including the agricultural, construction, and health sectors.

Moreover, the shortage of blood reserves in Spanish hospitals and the increase in immigrants who donate blood indicate a need for sensitive diagnostic techniques capable of detecting parasites in blood. Currently no tests are approved in Spain to screen donated blood for malaria, and careful questioning is essential to identify prospective donors at risk for transmitting malaria.

Experience in our Spanish reference laboratory indicates that new laboratory tests should be implemented to screen high-risk donor blood, especially for malaria and Chagas disease. These tests should be sensitive enough to reject all contaminated donations but specific enough to achieve a strong positive predictive value and minimize unnecessary deferral of otherwise acceptable donors.

We describe the potential use of the seminested multiplex malaria polymerase chain reaction (SNM-PCR) to screen potential blood donors at risk (immigrants from malaria-endemic areas) and reduce the time they are excluded as regular blood donors (3 years). We have reported that the SNM-PCR is capable of detecting 0.004 to 0.04 parasites per microliter of blood (6). In accordance with these data, we addressed the following questions: could SNM-PCR be a useful tool to screen prospective donors at risk? If so, could the 3-year deferral period be shortened for donors whose PCR results are negative?

To evaluate SNM-PCR in potential blood donors at risk, we conducted a study, approved by the Ethical Committee of the National Institute of Health, with blood samples from the Red Cross Blood Center in Madrid (Spain). Throughout 2000, our laboratory received 125 samples (5 $cm^3$ of whole blood in EDTA per sample) from blood donors at risk (56 from Colombia, 50 from Ecuador, 3 from Cameroon, 2 from Peru, 2 from Iran, 2 from China, and 1 each from India, Papua New Guinea, Sri Lanka, Mozambique, Mexico, Senegal, Costa Rica, Brazil, Kenya, and Tanzania). All donors at risk had recently (3 months to 1 year) immigrated to Spain.

Five samples (two from Ecuador, two Colombia, and one India) were positive for *P. falciparum* in two amplifications from two different blood extractions per sample. The five PCR-positive blood specimens were negative by microscopy (7) (we assumed that 400 microscopic fields are equivalent to 1 µL of blood). Our results suggest the following: a) the potential of this population to transmit malaria parasites was confirmed (4% of blood samples were positive), justifying the legislation in force (2,3); and b) SNM-PCR could serve as the reference test for screening donor blood, which could increase the use of these blood donations and consequently shorten the deferral period for blood donors.

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## Evidence Against Rapid Emergence of Praziquantel Resistance in *Schistosoma haematobium*, Kenya

**To the Editor:** The key issue in the development of drug resistance in parasitic helminths that do not multiply in their final host is the proportion of worms that remain in refugia (i.e., that are not exposed to the drug) relative to the number that are exposed but survive treatment (1). If the latter population is relatively large (as might occur, for example, after mass rather than targeted treatment), the worms that survive therapy could make a substantial contribution to the gene pool of the next generation, thus increasing the likelihood that resistance would develop. Since only a relatively small part of the population in Msambweni area of the Coast Province was treated by King et al. (2), it would be surprising if resistance had emerged.

If a predictive model is to work well, information should be available about the actual percentage of worms that already have genes for resistance. For example, in some communities in Kenya oxamniquine-resistant worms were relatively common before the drug had been used widely (3). The same may be true for praziquantel resistance in *Schistosoma mansoni* in Senegal (4-6). The large variation in response of *S. haematobium* found in field trials (Table 2 in [2]) suggests that genes for resistance to praziquantel could already be present in some areas. Until there are polymerase chain reaction probes for praziquantel resistance, the prevalence of genes for resistance to praziquantel could be estimated by giving two—or preferably three—treatments of praziquantel at monthly intervals and determining the reduction in egg counts after each round of treatment. Resistance could be confirmed through infection and treatment of rodents with isolates from uncured patients or by a simple test measuring the response of miracidia to praziquantel (7). With this information, it should be possible to make realistic predictions about the development of praziquantel resistance.

Although King and colleagues suggest the use of targeted treatment, it would perhaps be unfortunate if the optimistic-sounding title of their paper encouraged the mass use of praziquantel in the belief that resistance will not develop rapidly. This hope cannot be justified on the evidence presented.

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## Evidence Against Rapid Emergence of Praziquantel Resistance in *Schistosoma haematobium*, Kenya–Reply to Drs. Coles, Liang, and Doenhoff

**To the Editor:** Drs. Coles, Liang, and Doenhoff have raised important issues regarding the emergence of praziquantel resistance in human populations. We agree that praziquantel resistance will undoubtedly emerge. In our recent modeling paper (1), we attempted to address the question of how soon such resistance will become clinically significant.

As Dr. Cole and colleagues discuss, in the future the best means for detecting resistance will be through laboratory testing of field isolates for resistance genes. In the meantime, in the absence of validated laboratory testing, analysis of ongoing clinical experience provides *Schistosoma haematobium* control programs some useful insight into the potential emergence of drug resistance.

Our modeling analysis of the emergence of praziquantel resistance took as its base-case the 8-year experience with treatment outcomes in an area of Kenya that had not previously been exposed to praziquantel. It was not, in fact, "a relatively small part of the population" that was treated, but rather the greater majority (75%-95% per year) of all school-aged children in the Msambweni area. Based on the uneven age distribution of *S. haematobium* infection (2), we estimated that 50%-75% of worms in the community were exposed to the drug during the treatment period. Sensitivity analysis allowed our model to address the implications of greater or lesser worm exposure and of greater or lesser prevalence of resistance genes.

Clearly, untreated worms in refugia would have played an important role in delaying emergence of resistance during the study period; our analysis suggests that attempts to increase community treatment coverage to 100% would have accelerated the emergence of clinically significant resistance. Similarly, a higher initial prevalence of resistance gene(s) or a faster genetic mutation rate would be predicted to hasten the onset of substantial levels of resistance. Still, on the basis of known features of parasite transmission dynamics, the effects of obligate sexual parasite reproduction and of worm clustering within human hosts were predicted to slow the emergence of resistance (on a population basis) by several years. We agree that Dr. Van Wyk's recent review on "Refugia" (3) provides a thought-provoking discussion of the effects of mass treatment of helminthic infections in a setting where drugs are not 100% effective in eradicating infection, where transmission quickly resumes, and where reinfection with resistant parasites is favored.

The title of our paper was not meant to cast doubt on the likelihood of praziquantel resistance. Instead, it was meant to point out that, under the conditions of our study, we observed no substantial praziquantel resistance and its emergence was not as "rapid" as might have been predicted. We concur that the spread of resistance will be accelerated

by widespread drug usage, and we emphasize that targeted treatment has the potential advantage of prolonging the useful lifespan of a drug such as praziquantel.

The conclusion of our modeling analysis is that there may be only a 7- to 10-year period during which control projects will consistent, drug-mediated reductions in worm burden. It is essential, therefore, that planners anticipate eventual drug failure and incorporate, as part of an integrated infection-management system, nondrug interventions that will prolong drug usefulness. Prevention of transmission and not just development of newer drugs will finally provide the best form of "therapy."

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## Reiter Syndrome Following Protracted Symptoms of *Cyclospora* Infection

**To the Editor:** I read with interest and some dismay the report by Connor et al. on Reiter syndrome following protracted symptoms of *Cyclospora* infection (1). Wallace and Weisman summarized quite eloquently the history of "Reiter's syndrome" (2). It is now well documented that the syndrome had been described several hundred years before Reiter's publication. More importantly, Hans Reiter was a war criminal, having participated in or supervised medical "experiments" conducted on concentration camp inmates by the Nazis. Wallace and Weisman suggest "Reiter does not deserve eponymous distinction. The disorder should be renamed 'reactive cutaneo-arthropathy,' or 'reactive arthritis' syndrome." I agree with this proposal, have made it my practice, and urge this journal and my colleagues to do the same.

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## Report Summary

### Biological Warfare

The following is a summary of testimony presented by Joshua Lederberg on August 24, 2001, to the Committee on Foreign Relations, Senator Joseph R. Biden, Chair. This testimony is particularly pertinent in view of the recent terrorist attacks and subsequent episodes of anthrax.

Dr. Lederberg, a research geneticist, is past president and Sackler Foundation Scholar, The Rockefeller University. He was a pioneer in the field of bacterial genetics with the discovery of genetic recombination in bacteria. In 1958, at the age of 33, Dr. Lederberg received the Nobel Prize in Medicine for this work and subsequent research on bacterial genetics. Since 1966, he has been concerned about the potential abuse of microbiology and has advised government agencies about its control. In 1989 he was honored with the National Medal of Science by President George H.W. Bush, who cited his role as consultant. Dr. Lederberg is cochair of a study on biological weapon threats and defensive measures tasked by the Defense Science Board and the Defense Threat Reduction Agency. He is also the editor of "Biological Weapons: Containing the Threat," published by MIT Press in 1999.

I am honored to address the committee on a matter of transcendent importance to U.S. security and global human welfare. I define biological warfare as use of agents of disease for hostile purposes. This definition encompasses attacks on human health and survival and extends to plant and animal crops. Biological warfare was the focus of billion-dollar investments by the United States and the former Soviet Union until President Nixon's unilateral abjuration in 1969. This declaration was followed by the negotiation, ratification, and coming into force (in 1975) of the Biological Weapons Convention, a categorical ban on the development, production, and use of biological weapons.

Biological weapons are characterized by low cost and ease of access; difficulty of detection, even after use, until disease has advanced; unreliable but open-ended scale of predictable casualties; and clandestine stockpiles and delivery systems. Per kilogram of weapon, the potential lives lost approach those of nuclear weapons, but less costly and sophisticated technology are required.

Intelligence estimates indicate that up to a dozen countries may have developed biological weapons. Considerable harm (on the scale of 1,000 casualties) could be inflicted by rank amateurs. Terrorist groups, privately or state-sponsored, with funds up to $1 million, could mount massive attacks of 10 or 100 times that scale. For each 1,000 persons on the casualty roster, 100,000 or 1,000,000 are at risk and in need of prophylactic attention, which in turn necessitates a massive triage. Studies of hypothetical scenarios document the complexity of managing bioterrorist incidents and the stress that control of such incidents would impose on civil order.

While powerful nations maintain a degree of equilibrium through mutual deterrence and shared interests, less powerful elements may find in biological warfare opportunities to harm their enemies. Under current levels of preparedness (e.g., physical facilities and organization and operational doctrines), biological warfare is probably the most perplexing and gravest security challenge we face.

President Nixon's abjuration of biological warfare as a U.S. military weapon in 1969 set in motion the most important diplomatic and legal steps towards its eradication globally, laying the groundwork for the Biological Weapons Convention treaty. The treaty lacks robust verification mechanisms, mainly for reasons intrinsic to the technology. However, verification is not the foundation of the U.S. stance; the United States has long since abandoned the idea that it would respond in kind to such an attack. Were it not for the Biological Weapons Convention, a gradually escalating technology race would have amplified even further this threat to human existence. The treaty does set a consensually agreed-upon standard of behavior: it has become institutionalized into international law, and infractions open the door to enforcement.

Although further provisions for verification would do little to enhance our knowledge of those infractions, they would nevertheless have important symbolic value in reaffirming international commitment to the principles of the treaty. Creative leadership is needed to develop other ways to strengthen that reaffirmation. The real problem with the Biological Weapons Convention is enforcement, not verification. We have all-but-certain knowledge that Saddam Hussein has continued Iraq's biological weapons development program. To convince our allies, much less neutral nations and potential adversaries, of what is at stake, we may have to elevate the priority we give to this threat. We must also become more knowledgeable about the local political and cultural terrain and more ingenious in designing sanctions that will not impose undue hardship on the Iraqi population. Our public diplomacy is predicated on the stated proposition that use of biological weapons is an offense to civilization. This major accomplishment of the Biological Weapons Convention needs to be reaffirmed both in the attention we give to our own defense and in our stern responses to substantial infractions from any quarter.

Unlike the aftermath of nuclear or high-explosive bombardment, attack with biological weapons is amenable to interventions for some hours or days after the event, depending on the agent used. With the most publicized agent, anthrax, administration of appropriate antibiotics can protect the majority of those exposed. The other side of the coin is recognizing the syndrome within hours of the earliest symptoms. Biosensors are being developed to confirm suspicions of anthrax. We will have to rely on early diagnosis of the first human (or animal) cases to provide the basis for focusing those sensors. Because a wide list of diseases must be considered, this surveillance entails reinvigorating our overall public health infrastructure. In contrast to the explosive rise of health-care expenditures, public health funding has been allowed to languish, boosted only very recently by public arousal about emerging infections and bioterrorism. That boost entails personnel and organizational structures, but improvement also depends on funding for new as well as established programs.

In addition to diagnostic capability, we need organizational and operational doctrines that can confront unprecedented emergencies, we need trained personnel on call, and we need physical facilities for isolation, decontamination, and care. We also need stockpiles of antibiotics and vaccines appropriate to the risk, preceded by careful analysis of what kinds and how much. We need research on treatment methods (e.g., how should inhalational anthrax be managed with possibly limited supplies of antibiotics). Still more fundamental, research could give us sharper tools for diagnosis and more usable ranges of antibacterial and antiviral remedies.

Organizing the government to deal with mass contingencies is a goal that is vexing and still poorly addressed. It entails coordination of local, state, and federal assets and jurisdictions and the intersection of law enforcement, national security, and public health. A time of crisis is not ideal for debates over responsibility, authority, and funding.

Our main bulwark against direct large-scale attack is the combination of civic harmony and firm retaliation. Better intelligence is key to retaliation, apprehension, and penal containment and sanctions. This territory is technically unfamiliar to most of the intelligence community, which has taken many positive steps but has a long way to go.

Resources for managing biological threats are fewer than those allocated to other, more familiar threats.

I have already alluded to public diplomacy (starting with firm conviction at home) about the level of priority to be given to the biological weapons threat if a successful attack is to be averted. A dilemma is how to study the threats of biowarfare in detail and develop vaccines and other countermeasures, while maintaining the policy of abhorrence at the idea of using disease as a weapon. The central premise of the Biological Weapons Convention is that infectious disease is the common enemy of all humans and that joining with that enemy is an act of treason against humanity. This premise clearly inspired adherence to the Convention, even by countries that might otherwise exploit biological weapons to level the playing field against a superpower. Having set aside biological weapons as of small advantage to U.S. military power, we are fortunate that we share the treaty's interests and conclusions. They can only be strengthened if we internalize them and participate ever more fully in global campaigns for health. Current levels of funding for AIDS, malaria, and tuberculosis are small but are certainly steps in the right direction. We should assume leadership among nations cooperating with the World Health Organization to bolster global systems of surveillance and outbreak investigation of diseases that could threaten us all.


## International Conference on Emerging Infectious Diseases, 2002

The National Center for Infectious Diseases, Centers for Disease Control and Prevention, has scheduled the Third International Conference on Emerging Infectious Diseases for March 24-27, 2002, at the Hyatt Regency Hotel, Atlanta, Georgia, USA. More than 2,500 participants are expected, representing many nations and disciplines. They will discuss the latest information on many aspects of new and reemerging pathogens, such as *West Nile virus* and issues concerning bioterrorism.

Conference information is available at http://www.cdc.gov/iceid

The Call for Abstracts is available at http://www.asmusa.org/mtgscr/iceido2.htm

Contact person is Charles Schable, cas1@cdc.gov

## OPPORTUNITIES FOR PEER REVIEWERS

The editors of Emerging Infectious Diseases seek to increase the roster of reviewers for manuscripts submitted by authors all over the world for publication in the journal. If you are interested in reviewing articles on emerging infectious disease topics, please e-mail your name, address, qualifications or curriculum vitae, and areas of expertise to eideditor@cdc.gov

At Emerging Infectious Diseases, we always request reviewers' consent before sending manuscripts, limit review requests to three or four per year, and allow 2-4 weeks for completion of reviews. We consider reviewers invaluable in the process of selecting and publishing high-quality scientific articles and acknowledge their contributions in the journal once a year.

Even though it brings no financial compensation, participation in the peer-review process is not without rewards. Manuscript review provides scientists at all stages of their career opportunities for professional growth by familiarizing them with research trends and the latest work in the field of infectious diseases and by improving their own skills for presenting scientific information through constructive criticism of those of their peers. To view the spectrum of articles we publish, information for authors, and our extensive style guide, visit the journal web site at www.cdc.gov/eid .

For more information on participating in the peer-review process of Emerging Infectious Diseases, e-mail eideditor@cdc.gov or call the journal office at 404-371-5329.

## The Cover

### Cover of Le Vie d'Italia magazine from 1924

**Provided courtesy of Dr. Guido Sabatinelli.**

Le Vie d'Italia was established near the end of the 19th century in Milan as the monthly magazine of the Touring Club of Italy. Some 30 years later, in the 1920s, the magazine took on the additional role of Bulletin of the (then informal) National Organization for Tourism in Rome. Today, the magazine, which has been very popular, is still published by the Touring Club of Italy under the name Quì Touring.

The issue of Le Vie d'Italia featured on this cover of Emerging Infectious Diseases is from August 1924 (Year XXX, No.8, circulation 150,000). The Italian text reads, "Monthly Magazine of the Touring Club of Italy, Milano, 10 Corso Italia–Roads of Italy, Official Bulletin of the National Body of Tourist Enterprises."

In the center of this Le Vie d'Italia cover, under the image of the mosquito looming over the water, the inscription reads, "From the painting by E. Serra, Evening in the Pontine Marshes." The Pontine marshes, 50 Km south of Rome, were at that time the most malarious area of continental Italy–today the area is one of the most fertile plains in the country.

In the lower part of the cover, the Italian text reads, "Esanofele tablets, Esanofelina syrup for children, against malaria fever." Esanofele was produced by the F. Bisleri Company, which also produced a famous liqueur, Ferrochina Bisleri, probably an alcohol infusion of cinchona bark, herbs, and iron salts. In 1924, quinine was produced only under a state monopoly, so Esanofele was probably also an herbal preparation with perhaps some antimalarial activity due to the cinchona bark.

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# 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 Reviews, and Dispatches. 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 homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

# Instructions to Authors

## Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

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). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Arial. Mac files should be sent in MS Word or RTF formats.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in 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 in full. List the first six authors followed by "et al."

Tables and figures. Create tables within MS Word's table tool. Do not format as columns or tabs; do not submit tables in Excel. For figures, use color as needed. Send digital files (see above) or camera-ready slides, photographs, or laser prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Access the journal's style guide at http://www.cdc.gov/ncidod/EID/style_guide.htm

## Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov

# Types of Articles

**Perspectives, Synopses, Research Studies, and Policy Reviews**
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.

**Perspectives:** Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or 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**: 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. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

**Research Studies:** These articles 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 (e.g., "Here is what we found, and here is what the findings mean").

**Policy Reviews:** Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

**Dispatches**: These brief articles 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. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

**Another Dimension:** Thoughtful essays on philosophical issues related to science and human health.

**Book Reviews:** Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

**Letters:** This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**News and Notes**: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.