The Quarantine of Dubrovnik

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Scientists studying zooplankton population dynamics at a salmon hatchery, Loch Duich, Scotland.
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Shipping and the Spread of Infectious Salmon Anemia in Scottish Aquaculture

Alexander G. Murray,*† Ronald J. Smith,* and Ronald M. Stagg*

Long-distance transport of pathogens plays a critical role in the emergence of novel diseases. Shipping is a major contributor to such transport, and the role of ships in spreading disease has been recognized for centuries. However, statistical confirmation of pathogen spread by shipping is usually impractical. We present evidence of invasive spread of infectious salmon anemia in the salmon farms of Scotland and demonstrate a link between vessel visits and farm contamination. The link is associated with vessels moving fish between sites and transporting harvest, but not with vessels delivering food or involved in other work.

Anthropogenic activity increases the incidence of infectious diseases, which in turn influence the populations and production of marine organisms (1), from free-living bacteria (2) to mammals (3). The aquaculture industry has been strongly affected by diseases emerging from anthropogenic activities, and itself has played a critical role in their spread (4).

Infectious salmon anemia is an emerging disease causing severe damage to the salmon-farming industry in an increasing number of countries. The disease, first reported in Norway in 1984 (5), has since been reported in Atlantic Canada (1996) (6); Scotland (1998) (7); the Faroe Islands and possibly Chile (1999) (8); and most recently Maine, USA (late 2000) (9); over the last few months (2001), infectious salmon anemia has spread rapidly in Maine (10). In 1999, the annual cost of infectious salmon anemia was reported to be US$11M in Norway and US$14M in Canada, while in Scotland, the total cost of the epidemic of 1998-99 was US$32M (11). It is too early to say what the cost of the disease will be in Chile and the United States, but both countries have large salmon-farming industries. In almost all cases, infectious salmon anemia has mainly affected Atlantic salmon, *Salmo salar*, but in Chile, deaths have been reported among Coho salmon, *Oncorhynchus kisutch* (8). All deaths have been among farmed salmon.

Infectious salmon anemia is caused by novel virulent strains of a virus that has adapted to intensive aquacultural practices and has exploited the associated traffic to spread both locally and internationally (12). Genetic (13,14) and phenotypic (15) differences suggest that this adaptation occurred independently in Europe and the Americas. The virus strains then aggressively expanded their geographic ranges.

Invading new areas is critical for the spread of exotic species (16), including pathogens (12,17). Shipping has been identified as a major factor in movement of exotic species to coastal regions (16,18). The role played by ships in the introduction and spread of Black Death (a virulent form of plague) in 14th-century Europe has been extensively chronicled (19).

Recently, huge numbers of bacteria (8.3 x 10^8 L^{-1}), including *Vibrio cholerae,* the agent of cholera, and viruses (7.4 x 10^9 L^1) have been detected in ballast water of ships entering U.S. waters (17). Given the increasing volume of shipping (18), introduction of pathogens to coastal ecosystems is likely to increase. The rapidly growing aquaculture industry, with its high densities of potential host monocultures, is based in such coastal ecosystems (4).

Numerical analysis of the role of shipping in spreading pathogens is usually not possible because of heavy ship traffic and the multitude of pathogen sources. In this article, we examine the role of shipping in the invasive spread of infectious salmon anemia among Scottish salmon farms.

The Study

Infectious Salmon Anemia Virus (ISAV) in Scotland

In May 1998, the previously exotic viral disease infectious salmon anemia was detected at a salmon farm in Loch Nevis on the Scottish west coast (7,20,21) (Figure 1). ISAV is an orthomyxovirus of a new genus (*Aquaorthomyxovirus*) that is closely related to the influenza viruses (22). ISAV subsequently spread to salmon farms throughout Scotland. Molecular epidemiologic studies indicate that a single strain was responsible for the initial epizootic (14), although a second strain unrelated to the outbreak was later identified (23). Infectious salmon anemia has been reportable in the United Kingdom since 1990 (24), so its spread has been well surveyed and documented. The pattern of spread was discontinuous, with farms in Shetland becoming infected during summer 1998 but ISAV detected in Orkney in late 1999. Many intervening areas did not become infected, and in many cases, farms close to infected sites did not become infected (20). The broad area of infection with multiple isolated foci is not consistent with diffusive spread of disease by fish-to-fish contact or through vectors such as parasites or seabirds (25,26); this is not to say that this form of spread may not have occurred occasionally or locally in the vicinity of outbreak foci.

*Fisheries Research Services Marine Laboratory, Aberdeen, United Kingdom; and †University of Aberdeen, Aberdeen, United Kingdom
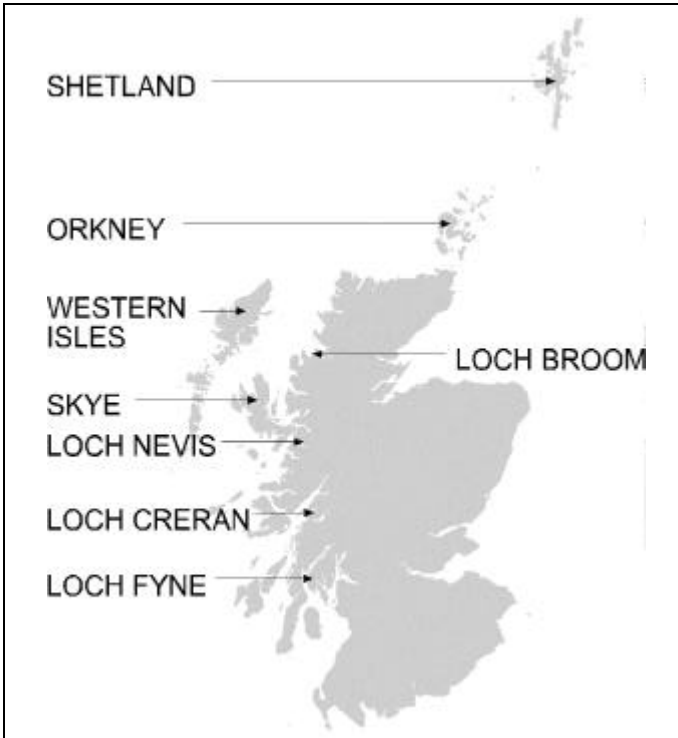
Figure 1. Map of Scotland showing locations named in text. The lochs shown are marine fjords.

Because of the broad pattern of spread, the movement of live fish and contaminated equipment between fish farms was suspected to play a role in the spread of this disease within Scotland (20). In aquaculture, salmon are moved extensively. They are reared at freshwater hatcheries, transferred to (and sometimes between) marine production sites, and finally transported to central harvesting and processing stations. In particular, well boats, which are used for the transport of fish, supplies, and equipment between farms and as work platforms, were suspected in the spread of ISAV (20).

Well boats and ferries have been suggested as possible routes of introduction of otherwise unexplained outbreaks of infectious salmon anemia in salmon farms in Norway, where the disease has been established since 1984 and has infected 101 fish farms in 1990 alone (27).

We conducted a quantitative analysis of the spread of ISAV in Scotland and its relationship to the movements of four well boats that serviced the farmed-salmon harvesting center of Loch Creran in the year preceding the epidemic (May 1997 to May 1998). These well boats made 1,558 visits to fish farms along >850 km of coastline from Loch Fyne to Shetland. Farms not visited by these well boats are not included in this analysis (including in Orkney and the Western Isles, where ISAV was later detected). These farms were serviced by other well boats, for which movement data were not available. We grouped the visited farms into 26 areas since individual fish farms are not always identifiable from the boats' logs. Of these areas, 6 were infected (28), 7 were suspected of being infected, and 13 escaped infection. Infectious salmon anemia was con-

firmed if clinical disease was present and ISAV was identified as the causal agent by both pathologic lesions and the presence of virus (28). In areas where infection was suspected, ISAV was identified by polymerase chain reaction (PCR) analysis (29) or similar immunofluorescence (30) methods or by clinical signs without confirmation of infection (28). Usually two of these three methods were used to confirm suspected status. A histogram of the number of areas included in these three categories clearly showed a relationship between the log number of boat visits and the risk for infection (Figure 2).

In two areas, infection developed after only a few visits. Loch Broom was suspected of being infected after a single visit by one of the boats. This infection occurred in 1999 and was therefore not part of the initial invasion. Infection also occurred on the Isle of Skye, where only two well-boat visits were recorded; however, in this case, fish stocks were moved directly from the infected site at Loch Nevis before infection was detected. Similarly, infectious salmon anemia was introduced to Shetland (21) when a partial load of smolts was brought to the Skye site, contaminating the load intended for shipment to Shetland. Since were direct introductions of infected fish to these sites, we excluded them from most analyses.

The harvest-processing station at Loch Creran used by the four vessels is also excluded from further analysis. Visits to this processing station, where fish were unloaded and held temporarily in net pens before slaughter, were qualitatively different from harvesting visits to other sites, where fish were grown. The fish at Creran were from multiple sources, while fish at other sites were from one or a few sources. Because all harvest trips ended at the processing plant, its inclusion would lead to double counting of harvest trips. The exclusion of Creran is particularly important for analyzing the efficiency of infection transfer during different types of visits.

Results

The quantitative nature of the relationship between visits and infection status is determined by scoring areas that did not become infected as 0, areas with suspected infection as 1, and
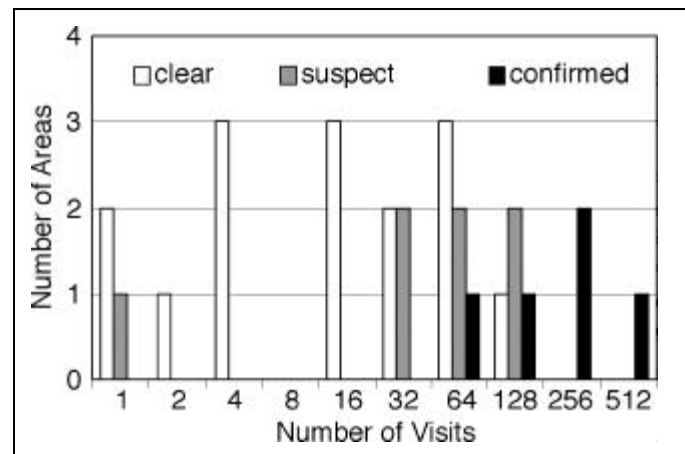
Figure 2. Distribution of infection by frequency of well-boat visits to salmon farms, Scotland.
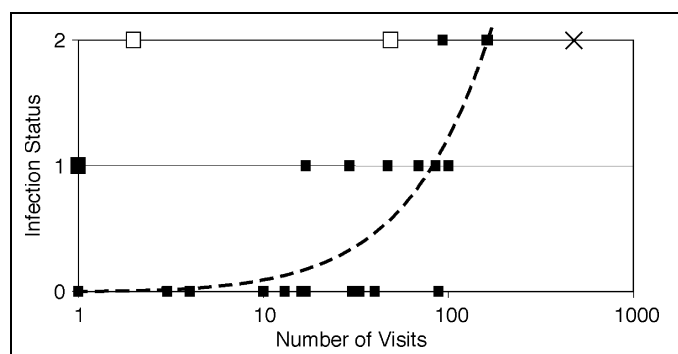
Figure 3. Infection status of areas versus number of well-boat visits. Infection status is 0 for no infection, 1 for suspected infection, and 2 for confirmed infection. Skye and Shetland, infected by fish transferred from Loch Nevis, are shown as large hollow squares, while the unconfirmed infection at Loch Broom is shown as a large filled square. Loch Creran is shown as a cross and is excluded to prevent double counting of harvest transport voyages. Regression is 0.012 x visits, $r^2 = 0.66$, p = 0.000004.

areas that did become infected as 2 (Figure 3). There is a significant relationship (regression of 0.010) between the number of visits and infection status ($r^2 = 0.23$, p = 0.0015). However, if regions where infection is explained by the movement of infected fish (Skye and Shetland) and Lochs Broom and Creran are excluded, the relationship between number of visits and infection status becomes much more statistically significant (0.012 regression) ($r^2 = 0.66$, p = 0.000004).

Well-boat logs divide vessel visits into three categories: shipment of harvest to Loch Creran, movement of live fish between other sites, and visits for general work plus delivery of food. We conducted a multivariate analysis of infection status versus these three categories of visits (Table); distance from Loch Creran is also included, showing that it is the number of these trips, not their length, that influences the spread of infection.

The multivariate analysis shows that infection status is not related to distance from Loch Creran or movement of well boats for general purposes. Infection is transferred only by the shipment of live fish or visits to the harvesting site. If the sites

infected by the movement of live fish from Loch Nevis and the uncertain site of Loch Broom are excluded, fish movements are also not related to infection status. Infected sites whose cause of infection is not explained by direct movement of infected fish are thus very strongly related to the number of harvest visits (p = 0.0009).

Harvesting involves transfer of live fish to Loch Creran, which is probably how this harvest-processing station became infected. At the time, the processing plant adjacent to the harvesting site was discharging effluent that had not been disinfected. Movement of the well boats from Loch Creran may then have rebroadcast ISAV.

Analysis of regional infection patterns within 50 km of Loch Creran shows a pattern of infection similar to the national pattern in that only movement of harvest vessels correlates with area infection status and r^2 is similar at 0.62. However, the relationship is far weaker, only 12 points (p = 0.049), because fewer data are available. The regional pattern of infection excludes, among other areas, Skye, Shetland, and Loch Broom. At this regional scale, the processes and sensitivities of transmission do not appear to differ much from those at the national level.

At the smaller scale of spread of within a few kilometers, which the coarsely resolved available data cannot resolve, other processes may have become important, including physical transport of virus by currents (associated with waste products [5,27]), escaped or wild fish (31), or vectors (such as sea lice) (32) moving between neighboring farms. Infected wild salmonids, which have been found throughout Scotland (33) could act as an ISAV reservoir (31).

The salmon farming industry and regulatory authorities responded to the epizootic by introducing strict controls on hygiene and on movements of well boats and live and dead fish and by making the slaughter of infected stocks compulsory. These measures resulted in the closure of the Loch Creran site (among others) and control of the major route of infection. In 1999, sites not visited by these well boats (and also Loch Broom, which was reportedly only visited once) became ISAV suspect (20). It is possible that once ISAV had become widely distributed, vessels other than those based at Loch Creran transported ISAV to new locations. Other transmission processes (e.g., sharing divers or equipment) (5,20) could also have played a role in disseminating the virus.

Conclusions

The evidence presented here supports a very strong quantitative link between the number of visits by well boats and the probability of ISAV detection in an area. The pattern of spread does not support a natural diffusive expansion of the ISAV epizootic, but the identical genetic nature of the ISAV at different sites indicates a direct link between incidents. Management activities could have resulted in interregional contact that coincided with the number of visits by well boats, but the strength of the relationship between well-boat visits and infection

Table. Relationship between type and distance of visit by well boats and infection status of salmon farm, Scotland[a]

	Harvest	Fish movement	General	Distance
I: $r^2 = 0.43$				
Relationship	0.011	0.021	0.0037	0.0006
p	0.03	0.09	0.65	0.59
II: $r^2 = 0.69$				
Relationship	0.014	0.009	0.0057	-0.00096
p	0.001	0.43	0.28	0.29
III: $r^2 = 0.62$				
Relationship	0.024	0.018	-0.0024	-0.010
p	0.05	0.41	0.82	0.44

[a]I: Excluding Creran only; II: excluding Creran, Loch Broom, Skye, and Shetland; III: within 50 km of Loch Creran (excluding Loch Broom).

implies that the well boats played a predominant role in transmission. This role is emphasized by the relationships between the specific type of visit and infection status. Simple exchange of equipment does not appear to increase risk for ISAV infection in Norway (5), and neither do general well-boat visits in Scotland. We therefore conclude that it is the movement of well boats, through shipment of live fish and visits for harvesting, that spread ISAV at the regional and larger (ranging from 10 to several hundred kilometers) scale in Scotland during 1998-99.

No new incidents of clinical infectious salmon anemia occurred in salmon farms in Scotland in 2000, and infection did not develop in new areas, although more farms reported isolated ISAV-positive reverse transcription PCRs. ISA also emerged in or was spread to the North Atlantic Faroe Islands. ISAV (but not infectious salmon anemia) was detected by PCR methods in wild salmonids throughout Scotland in 1999 (33), but although present, was much less prevalent in 2000 (21). The rapid response of the industry controlled the disease in farmed salmon and may have prevented infectious salmon anemia from becoming endemic in Scotland as it has in Norway (5,27).

After the introduction of stricter regulations in Norway in 1990 and 1991 to control hygiene in vessels and slaughterhouses, infectious salmon anemia outbreaks were reduced by nearly two orders of magnitude (27). This reduction shows that similar transport of poorly sanitized material by ships was also an important mechanism behind the infection's spread in Norway. However, infectious salmon anemia is established in Norway, and since 1991, its incidence has gradually increased again.

Our data suggest that well boats have played a major role in the spread of infectious salmon anemia in both Scotland and Norway. The pathogens carried by well boats had two possible sources: the processing plant, when the wells were unloaded and replacement ballast water was contaminated with processing plant effluent, or the adjacent harvesting station, when infected water was taken up. Alternatively, infected fish or fish detritus may have remained in the wells or pumps and pipework after fish were discharged to the harvest station and the vessel immediately left for a harvesting operation at another site. When well boats transfer between sites, they are inspected and disinfected (20), which should minimize the risk for infected cargo residue transferring ISAV. However, removal of all fish and residue from pumps and pipework is problematic (20). At the time of the epidemic, effluent from the Loch Creran processing plant was not fully disinfected, so ISAV could have been present in ballast water taken up after disinfection. When the well boat starts harvesting, this water is discharged so that fish can be loaded. It is difficult to assess the role of the two sources of pathogens in the shipborne transmission of infectious salmon anemia, although since pathogens are abundant in ballast water (17), the role shown for the harvesting station in broadcasting ISAV could have wider implications for the transmission of diseases by shipping. Infectious salmon anemia is only one of many emerging diseases present in marine environments (1); the aquaculture industry can both suffer from and assist in the spread of such diseases (4). While individual movements of potentially infected fish, particularly live stocks (34), processed or unprocessed carcasses used for fish food (1), or fish imported for human consumption (21), are most likely to spread disease, these movements can be monitored and controlled. Extensive ship traffic (18) and lack of regulation increase the risk of spreading disease to animals raised for aquaculture and to other animals in marine environments (4).

Diseases potentially spread by shipping include waterborne diseases of humans such as cholera (17) and potential viral zoonoses (35). Thus, although this article highlights anthropogenic spread of a pathogen economically damaging for aquaculture, it also underscores the potential role of shipping in the global transport of zoonotic pathogens.

Acknowledgments

Dr. Murray is senior research fellow at the FRS Marine Laboratory, Aberdeen, and the Department of Zoology, University of Aberdeen, Scotland. His research interests include modeling of diseases and parasites of farmed and wild fish and of other aquatic organisms.

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Address for correspondence: Alexander Murray, FRS Marine Laboratory, P.O. Box 101, Victoria Road, Aberdeen, AB11 9DB, Scotland, United Kingdom; fax: 44-1224-295-620; e-mail: murrays@marlab.ac.uk

Using a Dynamic Hydrology Model To Predict Mosquito Abundances in Flood and Swamp Water

Jeffrey Shaman, Marc Stieglitz, Colin Stark, Sylvie Le Blancq, and Mark Cane

We modeled surface wetness at high resolution, using a dynamic hydrology model, to predict flood and swamp water mosquito abundances. Historical meteorologic data, as well as topographic, soil, and vegetation data, were used to model surface wetness and identify potential fresh and swamp water breeding habitats in two northern New Jersey watersheds. Surface wetness was positively associated with the subsequent abundance of the dominant floodwater mosquito species, *Aedes vexans,* and the swamp water species, *Anopheles walkeri.* The subsequent abundance of *Culex pipiens,* a species that breeds in polluted, eutrophic waters, was negatively correlated with local modeled surface wetness. These associations permit real-time monitoring and forecasting of these floodwater and nonfloodwater species at high spatial and temporal resolution. These predictions will enable public health agencies to institute control measures before the mosquitoes emerge as adults, when their role as transmitters of disease comes into play.

I n their efforts to control mosquitoes and mosquito-borne diseases, public health officials would benefit if they could identify the locations of mosquito populations. Unfortunately, most public health agencies lack the resources for comprehensive sampling and monitoring of the spatial and temporal distribution of mosquito populations. In an effort to circumvent this shortcoming, researchers have attempted to account for fluctuations in mosquito populations through the monitoring of environmental conditions. Many such studies have associated the measured abundance of vectors or vector-borne disease incidence with satellite imaging (1-8). Such studies have generally used vegetation classification or the normalized differential vegetation index, which measures vegetation greenness, as proxies for soil moisture.

Patz et al. (9) estimated soil moisture more directly by using a dynamic hydrology model. Those researchers used a water balance model to hindcast weekly soil moisture levels in the Lake Victoria basin. (Hindcast is the retrospective prediction of historical conditions.) These soil moisture levels were then associated with local mosquito biting rates on humans and entomologic inoculation rates. This study demonstrated the potential application of dynamic hydrology models in epidemiologic monitoring; however, the model was coarse in both temporal and spatial resolution and lacked the means for assessing the spatial distribution of wetness across the land surface.

We present an example of how flood and swamp water mosquito abundance can be predicted in real time at high spatial resolution through application of a more detailed dynamic hydrology model (10). This model accounts for topographic variability and its control over soil moisture heterogeneity and runoff within a watershed. In doing so, the model resolves small areas of surface wetness and permits identification of the spatial distribution of potential breeding habitats within a catchment.

Our approach consisted of two components: physical and empirical. First, we used the dynamic hydrology model to hindcast the surface wetness (puddles, bogs, ponds) that potentially support floodwater and swamp water mosquito larvae. Then the spatial-temporal variability of this model-predicted surface wetness was empirically associated with the spatial-temporal variability of floodwater and swamp water mosquito abundances. We would have preferred to make this empirical association directly with larval collection data; however, because such data were not available, we established this association with adult mosquitoes collected in light traps. The result of this dynamic-empirical analysis is a logistic regression model fit relating local surface wetness to subsequent mosquito species abundances.

Modeling Overview and Methods

Surface wetness conditions are a function not only of precipitation and temperature but also of a number of other meteorologic variables controlling evaporation, as well as soil properties, vegetation, and antecedent conditions (prior wetness in the watershed). In addition, topography controls much of the spatial distribution of wetness in a watershed, i.e., valleys are generally wetter than the drier upland regions. Our hydrology model incorporates all these factors, using topography to account for the distribution of wetness across the land surface (10). The model tracks the expansion and contraction of lowland saturated zones within this spatial framework and generates a picture of the variable wetness of the land surface as it changes in time.

Columbia University, Palisades, New York, USA
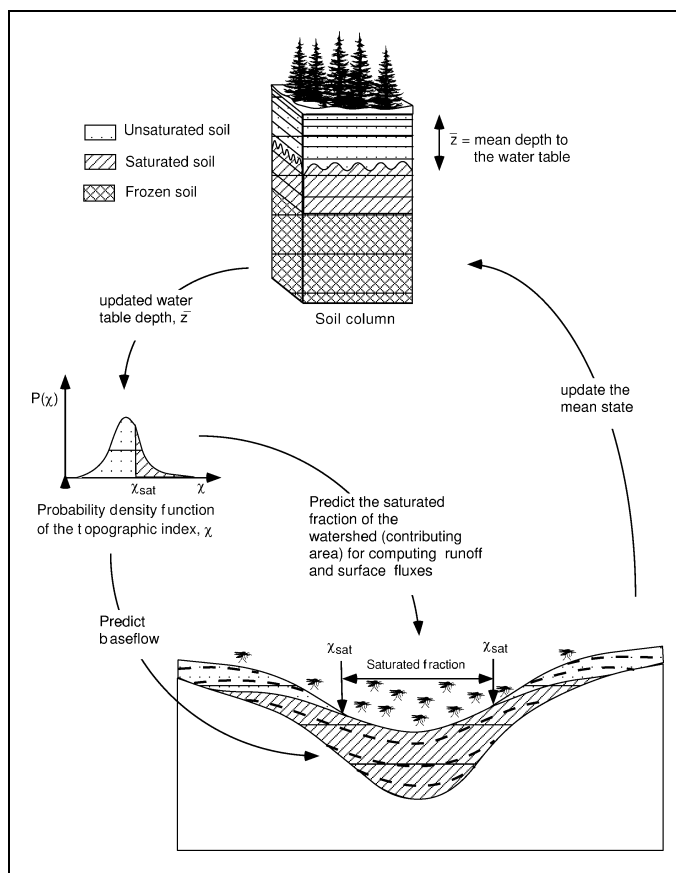
Figure 1. Schematic depiction of the hydrology model. The model couples the analytic form of TOPMODEL equations within a single column framework. From an update of the mean water table depth, TOPMODEL equations and Digital Elevation Model data are used to generate baseflow and the saturated fraction of the watershed.

For both large and small watersheds, it is possible to model surface wetness across individual plots of land as small as 100 m^2 (e.g., 10-m cells) and monitor how the surface wetness of these plots changes through time. We use an adaptation of TOPMODEL, a conceptual framework for rainfall-runoff modeling (11-14). Our model uses gridded digital maps of land surface topography and a dynamic numerical framework that

accounts for the movements of water and energy within the soil and at the surface. The mean depth of the water table and the statistics of topography within a watershed are used to compute the saturated areas of the watershed and the shallow groundwater flow that supports it (Figure 1). Thus, at any point in time we can create a mosaic of cells, each with a local model surface wetness, which taken as a whole represents the surface conditions of the entire watershed (Figure 2). This mosaic of surface wetness depicts the spatial variability of conditions at the land surface that result from terrain (topography, vegetation, and soil type) and integrated weather forcing (meteorologic conditions).

The modeling framework can be run continuously and has been validated at catchments ranging in scale from the Red Arkansas basin (570,000 km^2) (15) to the Imnavait Creek catchment (2.2 km^2) (16). Real-time surface wetness conditions can be generated by using current weather conditions. In addition, the hydrology model can be assimilated within the framework of global climate models, allowing integrated medium- to long-term forecasts of surface wetness conditions at a similar spatial resolution.

Application and Validation of the Hydrology Model

The probability distribution of the soil moisture deficit, i.e., statistics of topography, is generated from digital elevation model data by using a multidirectional flow routing algorithm, which is tied to an adaptive error correction (pit infill) scheme needed for low-relief areas such as coastal plains. Our algorithm is similar to that of Martz and Garbrecht (17).

The hydrology model is driven by an hourly record of air temperature, precipitation, relative humidity, surface pressure, wind speed, incident long-wave radiation, and solar radiation. Model simulation yields both hourly and daily time series of several catchment hydrologic variables, including mean water table depth (WTD), percent surface saturation, and total surface runoff. The last variable is used to generate a hydrograph for the outlet river of the catchment, which then can be compared with actual weir measurements.
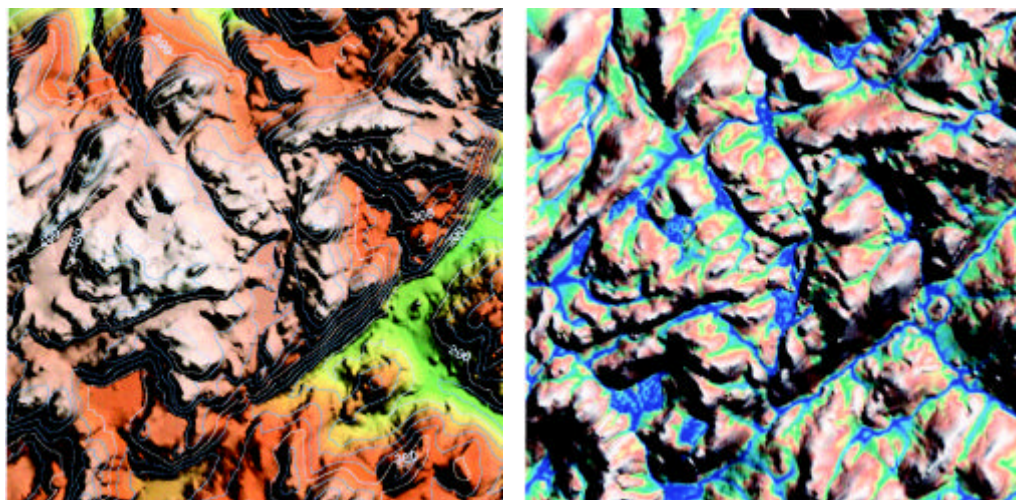
Figure 2. Visualization of the processing of land surface topography for a sample 25-km^2 area in New York State. a) Digital Elevation Model - a pixelated (10 m cells) representation of land surface topography. Contour lines (in meters) have been overlain. b) Postprocessing, depiction of land surface wetness at a single point in time. Blue areas are wettest. The variability and spatial distribution of surface wetness are evident.

Linking the Model to Mosquitoes

From the mean catchment WTD, a time series of local WTD can be calculated for any pixel within the watershed. Thus, the hydrology model links weather to changes in local surface wetness, including the saturated surface regions (i.e., pools and swamps) exploited by floodwater mosquitoes. The development time of these mosquitoes from egg until emergence as adult is 7 to 20 days, depending on species and temperature conditions (18,19). Because the model provides a time series of hydrologic variables affecting surface wetness at hourly and daily intervals, it captures the expansion and contraction of saturated surface regions (i.e., breeding pools) at rates that impact mosquito development.

Monitoring local WTDs also permits detection of mosquito breeding pools at very fine, subpixel, spatial scales. A modeled, local WTD for a given pixel of -0.2 m does not imply that the whole of the 30-m x 30-m pixel is dry, merely that the pixel mean elevation is. Given the variability in surface elevation–and in the water table depth—a percentage of the water table can be expected to outcrop. The shallower the local WTD for a given pixel, the greater the percentage of that pixel area that can be estimated to be wet at the surface. Thus, a pixel with a WTD of -0.4 m can be expected to have more surface pooling than a pixel with a WTD of -1.4 m. Both models and data have shown that substantial soil moisture heterogeneity exists at most scales within a catchment (20,21). This fractal geometry permits such extrapolation of the pixel-to-pixel variability of local WTDs to a smaller, subpixel scale. Hoof prints, ditches, tire tracks, and natural relief can all account for the heterogeneity of elevation within a pixel. Use of local WTD allows a statistical estimation of such potentially saturated portions of the surface.

Data Collection and Analysis

Two adult mosquito collection records from northern New Jersey were used: 1) a 13-year time series of daily mosquito counts (May through September) from seven sites near the Pequest River, and 2) a 15-year time series of daily mosquito counts (June through September) from a single site in the Great Swamp National Wildlife Refuge. (Collections at the Pequest River were not made every year at all seven sites; instead, records at these individual sites are from 1 year to 10 years of the full 13-year record.) Collections at each site were made with a New Jersey light trap and were counted and sorted by species. Trap locations were identified with a global positioning sensor. Figure 3 shows a representative 8-year time series of the floodwater species *Ae. vexans,* collected at the Great Swamp site.

Hourly meteorologic data were assembled from National Climate Data Center (NCDC) archives for nearby Allentown, Pennsylvania. Solar radiation data were provided by the Northeast Regional Climate Center (NRCC) from analysis of the NCDC data by the NRCC solar energy model (22). Catchment topographic statistics were generated from 30-m pixel resolution DEMs delineating the Pequest River and Great Swamp
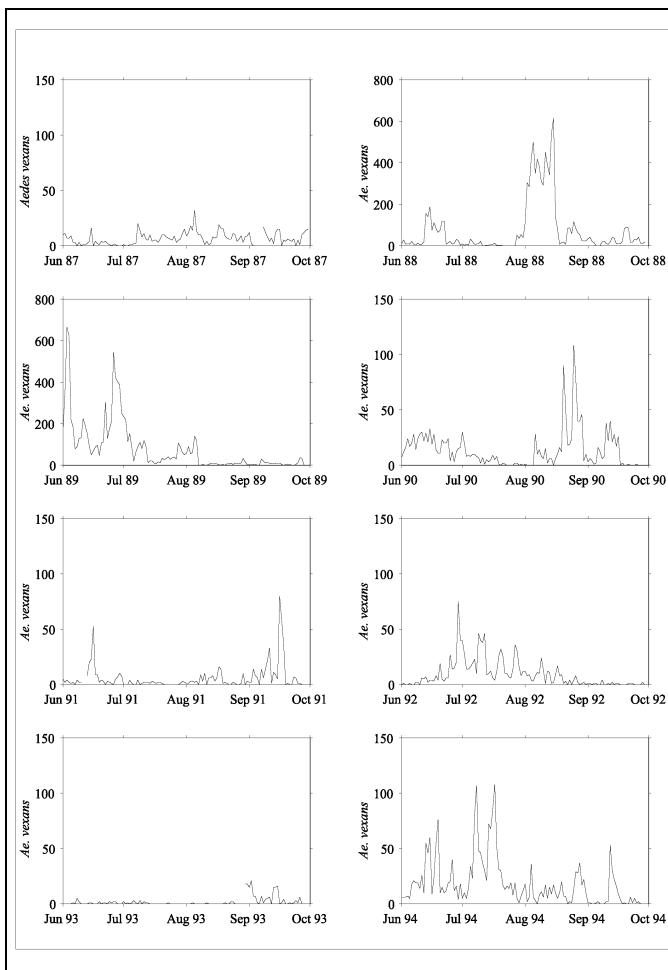
Figure 3. *Aedes vexans* collections at the Great Swamp, 1987–1994.[a]
[a]Considerable year-to-year variability is evident in these light trap collections. Note the different scaling for 1988 and 1989.

watersheds downloaded from the U.S. Geological Survey (USGS) website (23). The hydrology model was validated against USGS hydrograph data for the Pequest River catchment.

Two time series of daily mean catchment WTD were generated–one for the Pequest River catchment and one for the Great Swamp catchment. WTDs were then used to create indices of local wetness (ILWs), time series measures of the surface wetness of the immediate area surrounding each of the seven mosquito collection sites in the Pequest River catchment and the one site in the Great Swamp catchment. At each mosquito collection site, an ILW was constructed by using a square block of pixels–24 pixels on a side, centered about the geocoordinates of the site. Instead of elevation, each pixel was represented by its soil moisture deficit value. Using this square grid of pixels and the daily time series of mean catchment WTD, we computed the local WTD for each pixel at each daily time step. A threshold depth was then chosen (e.g., 0 m or 0.5 m; analyses were insensitive to this range of depth values). Every pixel in the matrix was then assigned a new value: one if the WTD for the pixel were higher than the chosen depth value or zero if the WTD for the pixel were deeper than the chosen

depth. This created a binary 24-by-24 matrix of ones and zeros at each daily time step. These numbers were summed for each time step to create an ILW, a time series array of values representing the local wetness of a collection site.

Statistical analyses were then used to estimate the association between modeled surface wetness–i.e., the ILW and mosquito species prevalence. Time series regression analyses were used at each site to determine the tendency and strength of the association between mosquito abundances and the collocated ILW. The time series regressions were performed by using the log (mosquitoes + 1), which normalized the collection data. Lag correlations were adopted to represent the average time of mosquito development from ovipositing until emergence.

From a public health perspective, it is often the mass emergence of mosquitoes that criticially needs to be identified. We used logistic regression to explore the ability of modeled surface wetness (the ILW) to predict the timing of such events. Logistic regression analyses were used to estimate the probability of a mass emergence, which we defined as a single-day collection of either $\geq$128, or $\geq$32 mosquitoes of a given species, depending on collection abundance. Multiple logistic regression analysis was used to determine the probabilities that collected mosquitoes would exceed a range of values (powers of two).

Analyses at the Pequest River sites were confounded by mosquito control efforts–spraying of both larvicide and adulticide in the areas of mosquito collection. As a consequence, regression analysis was restricted to the dominant floodwater species of the area, *Ae. vexans*. Logistic regression analyses were performed only at the Great Swamp site–the area without mosquito control.

Results

Table 1 presents correlation coefficients based on time-series regression analyses of *Ae. vexans* at two Pequest River catchment sites: Bernaski, for which there was 1 year of mosquito collection data; and Youngs Island, for which there were 10 years of collection data. At the Bernaski site, 50% of the variance in adult *Ae. vexans* was explained by the ILW (r = 0.70, p <0.01) with a 10-day lag (Table 1). Ten days is approximately the mean time of development of *Ae. vexans* from egg until emergence as adult, the period in which pooled surface habitats are necessary for mosquito survival (24). Figure 4 shows the regression model fit for this site. At the Youngs Island site, there is notable year-to-year variability in the correlation of the ILW with the abundance of *Ae. vexans* 10 days subsequent (Table 1). 1987 is the only year for which the association is positive and statistically significant (p<0.01). 1987 was also the only year without mosquito control at the Youngs Island site.

No mosquito control took place at the Great Swamp, and light trap collections were greatest at this site. Table 2 presents correlation coefficients based on time-series regression analyses of the three most abundant mosquito species. For the full 15-year record, the ILW was positively correlated with a 10-

Table 1. Yearly correlation coefficients, two Pequest River sites[a]

Year	Correlation coefficient (r)	Sample size (n)
Bernaski site		
1987	0.7046[b]	128
Youngs Island site		
1987	0.3748[b]	134
1988	0.1497	137
1990	0.0090	138
1991	-0.1038	151
1993	0.1271	150
1994	-0.4666[b]	147
1995	-0.2294[c]	130
1996	0.0474	153
1997	0.0431	153
1998	0.0256	147

[a]Correlation coefficients based on yearly regression analyses of *Ae. vexans* at two sites in the Pequest River catchment: a) Bernaski and b) Youngs Island. The index of local wetness was constructed for a depth of 0.5 m. log (count + 1), lagged 10 days, and used in the regression analyses.

[b]p<0.01.

[c]p<0.05.

day lag (p<0.0001) to both *Ae. vexans* and *An. walkeri*, a swamp water species (25). The ILW was negatively correlated with a 10-day lag (p<0.0001) to *Cx. pipiens*, a species that breeds in more polluted, eutrophic waters (26). While these values are statistically significant, the ILW fails to explain >12% of the variance of any of the mosquito species analyzed.

At the Great Swamp site there is also notable year-to-year variability in the amount of variance explained by the ILW. *Ae. vexans* is significantly correlated with the ILW for only 9 of the 15 individual years; for 1986 and 1994, this association is negative. Similarly, *An. walkeri* is significantly correlated with
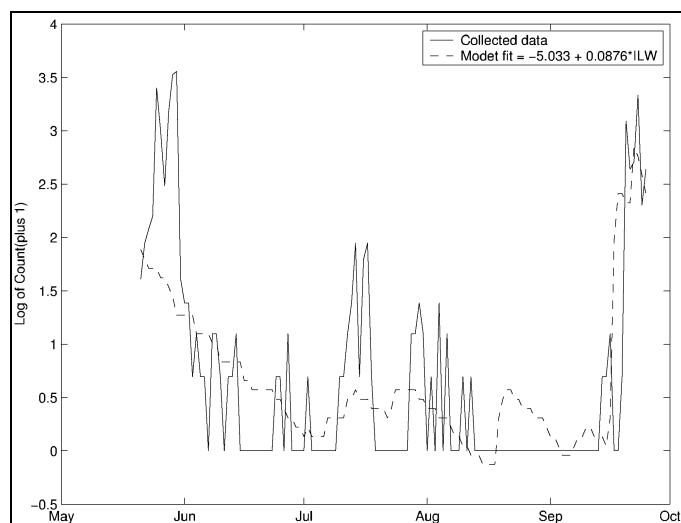
Figure 4. Time-series regression model fit of *Aedes vexans* 10 days later at the Bernaski site, Pequest River catchment. Regression fit is significant at p<0.01, r-squared = .50.

Table 2. Yearly correlation coefficients (r) Great Swamp site 10-day lag, log (count + 1) (p<0.05)[a]

Year	Aedes vexans	Anopheles walkeri	Culex pipiens
All 15 years	0.3433	0.2693	-0.2623
1984	0.5705	-	-
1985	0.3023	-	-
1986	-0.4186	-	-0.5698
1987	-	-	-0.2695
1988	0.2479	0.3380	-0.2888
1989	0.8018	0.4551	-0.6007
1990	0.5505	-	-0.2507
1991	-	-	-0.4271
1992	0.2022	-	-
1993	-	0.4502	-0.6108
1994	-0.4699	-0.7101	-0.6099
1995	0.5453	0.4047	0.2657
1996	0.5927	0.3445	0.2914
1997	-	-	-0.2172
1998	0.6376	0.5341	-

[a]Correlation coefficients based on full-record and yearly time-series regression analyses of the three most abundant species collected at the Great Swamp. The ILW was constructed for a depth of 0 m. log (count + 1), lagged 10 days, and used in the time-series regression analyses. Correlation coefficients for the full 15-year record are significant (p<0.0001). Yearly correlation coefficient values are listed only if significant at p<0.05.

the ILW for only 6 of 15 years examined, and for 1994, this association is also negative. While *Cx. pipiens* is negatively correlated with the ILW for the full 15-year record, only 9 of the 15 individual years have significant negative correlations; during 4 years the correlation was not statistically significant, and 2 years are significantly positively correlated (p<0.05).

Multiple logistic regression was performed on all 15 years of *Ae. vexans* data at the Great Swamp site. Figure 5 presents the results of this analysis; the model fitting demonstrates that with increasing local wetness the probability of more mosquitoes 10 days later increases significantly (p<0.0001). Logistic regression was also performed singly for mass emergence levels–daily collections of ≥128 *Ae. vexans*. Model fit was significant (p<0.0001) and yielded an equation for the probability of a mass emergence of *Ae. vexans* 10 days later (Table 3). Figure 6a depicts this relation; the probability of an *Ae. vexans* mass emergence increases exponentially at the wettest surface conditions.

Logistic regression were also performed for the other two abundant mosquito species at the Great Swamp site. The swamp water species, *An. walkeri,* was similarly positively correlated with ILW. *Cx. pipiens* was negatively associated with ILW. Light trap collections of both of these species were fewer than collections of *Ae. vexans*; therefore, for these species, mass emergence was defined as a daily collection of ≥32
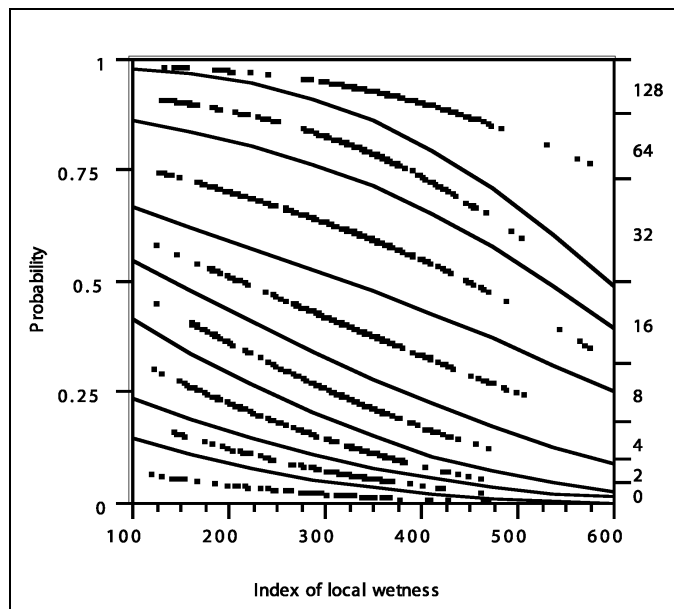
Figure 5. Logistic regression analysis of the complete 15-year record of Great Swamp site *Aedes vexans*. From bootom to top the solid lines represent threshold mosquito counts of increasing powers of two. Left vertical axis provides the predicted probability that the count of mosquitoes will lie at or below a given threshold. Surface wetness (the index of local wetness [ILM]) increases from left to right. Dots between lines illustrate the distribution of ILW values for mosquito counts falling between two successive threshold line values.

mosquitoes. Figures 6b and 6c show the probability of a mass emergence of *An. walkeri* and *Cx. pipiens*, based on these logistic regression analyses. The probability of a mass emergence of *An. walkeri* increases significantly with increasing local wetness (p<0.0001), whereas the probability of a mass emergence of *Cx. pipiens* decreases significantly with increasing local wetness (p<0.0001). Table 3 provides the model fit equations derived from these analyses.

Discussion

The methods used here provide the basis for a dynamic mosquito prediction system. This system is designed for non-urban settings and predicts different species abundances at high spatial resolution. For the northern New Jersey water-

Table 3. Logistic regression equations, Great Swamp site, p <0.0001[a]

Mosquito	Probability of a mass emergence
Aedes vexans	$p(\geq 128 \text{ Ae. vexans}) = (1+\exp(4.701 - 0.00804*\text{ILW}))^{-1}$
Anopheles walkeri	$p(\geq 32 \text{ An. walkeri}) = (1+\exp(6.362 - 0.0116*\text{ILW}))^{-1}$
Culex pipiens	$p(\geq 32 \text{ Cx. pipiens}) = (1+\exp(-0.353 + 0.00989*\text{ILW}))^{-1}$

[a]Equations for the probability of mass emergence (≥128 *Ae. vexans*; ≥32 *An. walkeri*; 32 or more *Cx. pipiens*) mosquitoes) 10 days later, based on logistic regression analysis. The ILW was constructed for a depth of 0 m. Model fits are significant at p <0.0001 based on a likelihood-ratio test.
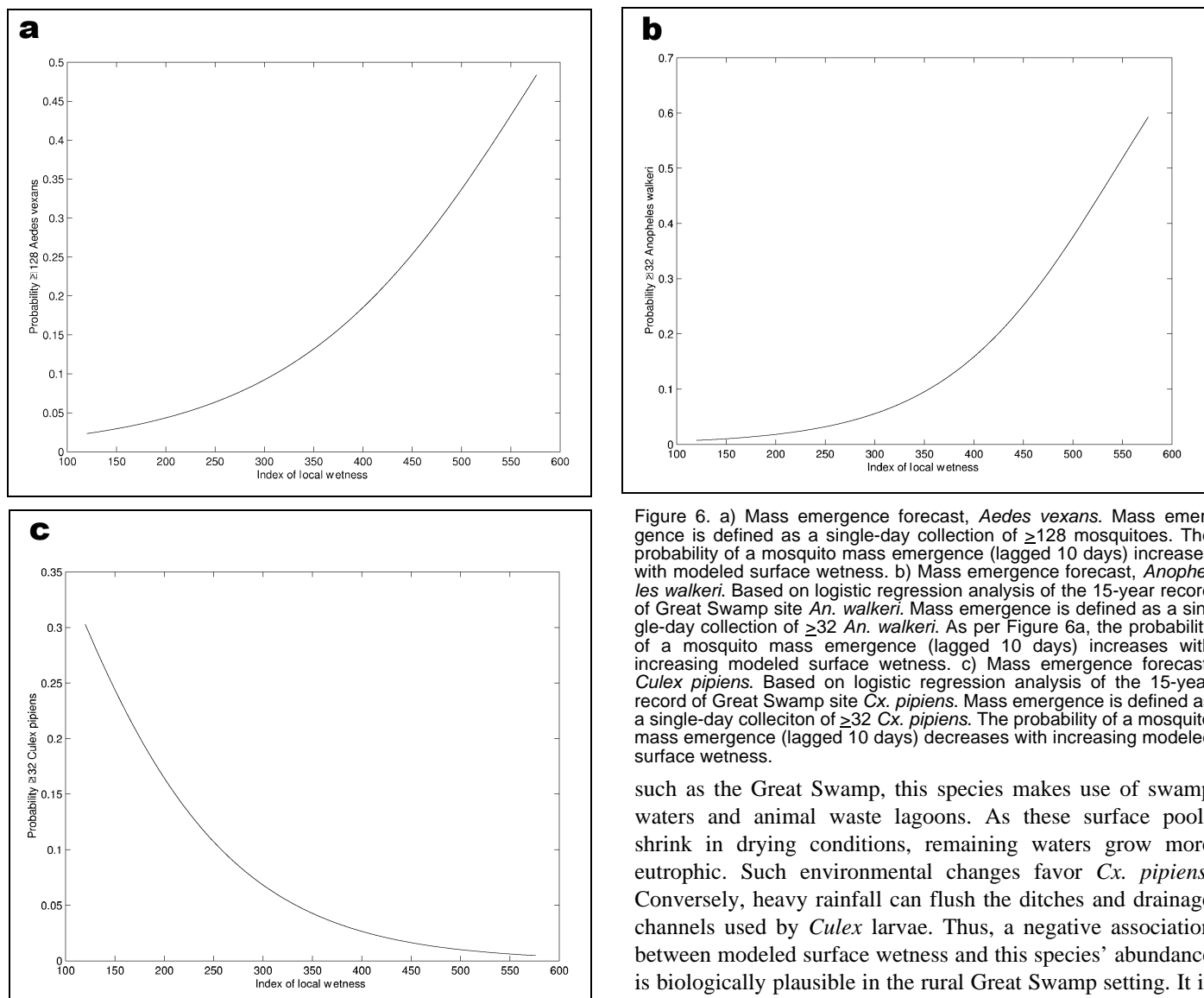
Figure 6. a) Mass emergence forecast, *Aedes vexans*. Mass emergence is defined as a single-day collection of $\geq$128 mosquitoes. The probability of a mosquito mass emergence (lagged 10 days) increases with modeled surface wetness. b) Mass emergence forecast, *Anopheles walkeri*. Based on logistic regression analysis of the 15-year record of Great Swamp site *An. walkeri*. Mass emergence is defined as a single-day collection of $\geq$32 *An. walkeri*. As per Figure 6a, the probability of a mosquito mass emergence (lagged 10 days) increases with increasing modeled surface wetness. c) Mass emergence forecast, *Culex pipiens*. Based on logistic regression analysis of the 15-year record of Great Swamp site *Cx. pipiens*. Mass emergence is defined as a single-day colleciton of $\geq$32 *Cx. pipiens*. The probability of a mosquito mass emergence (lagged 10 days) decreases with increasing modeled surface wetness.

sheds we examined, the flood and swamp water species, *Ae. vexans* and *An. walkeri*, were positively associated with local modeled surface wetness 10 days before. These findings quantify the link between surface wetness and mosquito abundance and suggest that prediction of the abundance of flood and swamp water mosquitoes is practicable. In particular, the logistic regression analyses at the Great Swamp site indicate that probabilistic forecasts of localized mass emergence potential are possible. If this correspondence is shown to be robust, it could be used as a forecast tool and aid in *Ae. vexans* and *An. walkeri* control efforts in New Jersey and surrounding areas.

Cx. pipiens abundance was negatively associated with local modeled surface wetness 10 days prior. This species is a vector of the *West Nile virus* (27) and St. Louis encephalitis (26,28). The negative association between modeled surface wetness and the abundance of *Cx. pipiens* is somewhat counterintuitive; however, there is reason to believe this finding is real. *Cx. pipiens* breeds in polluted, eutrophic waters (26). In rural settings,

such as the Great Swamp, this species makes use of swamp waters and animal waste lagoons. As these surface pools shrink in drying conditions, remaining waters grow more eutrophic. Such environmental changes favor *Cx. pipiens*. Conversely, heavy rainfall can flush the ditches and drainage channels used by *Culex* larvae. Thus, a negative association between modeled surface wetness and this species' abundance is biologically plausible in the rural Great Swamp setting. It is also consistent with studies alluding to an association between drought, eutrophication and *Culex* abundance (28). Further corroboration is provided by collections of a less abundant species, *Uranotaenia sapphirina,* with a similar life cycle (29), which was also negatively associated with modeled surface wetness (data not shown). These findings suggest that it may be possible to extend the model's forecast ability to nonflood-water species.

The logistic regression models developed here all include a 10-day lag between modeled surface wetness and subsequent mosquito abundance. Comparable associations between modeled surface wetness and subsequent mosquito abundance were usually found for lags ranging from 0 to 12 days (data not shown). This time-lag similarity is due in part to the slow variability of the water table, which fluctuates in height on time scales of weeks. The 10-day lag was chosen a priori to represent the average development time from oviposition to eclosion at the New Jersey sites for the mosquito species examined. We surmise that modeled surface wetness identifies

the location of larval habitats and that the mosquitoes collected 10 days later represent newly emergent adults from these larval habitats; however, without age-grade typing of the adult collections or larval sampling data, we lack direct support for this hypothesis.

Regression analyses demonstrated that year-to-year variability exists in the association between mosquito abundances and modeled surface wetness. This finding suggests that at this time a regression model is not the best means of predicting mosquito abundance; rather, a probabilistic forecast, developed from logistic regression analysis, is more appropriate. Further study of the year-to-year variability in the association of mosquito abundance and modeled surface wetness is required.

The ability to identify potential breeding habitats accurately in space and time, for even some vector species, will enable a greater measure of control over these vectors. To be sure, other factors affect disease transmission. Host immunity, pathogen ecology, host-vector-pathogen interactions, and the effects of temperature and humidity on mosquito development rates all would require consideration for a more complete understanding and quantification of a local disease system. Nonetheless, the presence or absence of an appropriate aquatic realm, necessary for egg, larval, and pupal development, is a critical determinant of mosquito species abundances (18,19,30). This dependence of vector species on aquatic habitats is an invariant aspect of the mosquito life cycle and one for which the energetics can be modeled explicitly by using the fundamental, physical equations of conservation of energy and mass. Additionally, mosquito-borne diseases require mosquitoes for transmission. Thus, the dependence of mosquitoes on aquatic habitats provides both a robust link between the biological and the physical realms and a logical starting point for the dynamic modeling of mosquito-borne disease systems, provided one bears in mind the protean nature of local disease systems.

The dynamic modeling presented here has several advantages over satellite imaging approaches: 1) it models the actual aquatic environment used by the mosquitoes, not a filtered proxy; 2) it offers continuous real-time prediction of mosquitoes; 3) it resolves the whereabouts of the potential breeding habitats at a very fine scale (areas as small as 10-m cells); and 4) the model is readily coupled to global climate models, allowing additional medium- and long-range forecast of mosquito abundances.

Use of a dynamic, numerical model also allows for broad application of its methods. Adjustment of the model from region to region and climate to climate is made by accounting for differences in topography, vegetation, soil properties, and weather conditions. With these considerations the hydrology model can be transplanted to any location. All that remains to be determined is the nature of the association of modeled surface wetness and local mosquito species population numbers. Thus, dynamic hydrology models have the potential to provide public health officials with flexible methods for the assessment of current and future vector population levels, and in addition, the ability to target control efforts based on the spatial distribution of these predicted populations.

Conclusion

We have presented methods for predicting mosquito abundance by using a dynamic hydrology model. Modeled local surface wetness was correlated with the subsequent abundance of three New Jersey mosquito species: *Ae. vexans, An. walkeri,* and *Cx. pipiens*. The sign and magnitude of the association of modeled surface wetness and species abundance appear to be a function of mosquito species biology and overall abundance. These correlations permit probabilistic forecast of mass emergences of these mosquitoes through the physical modeling of land surface hydrology.

Application of these methods across variable climate, biome, and species compositions will aid in wide-range monitoring of mosquito mass emergences through the modeling of surface wetness levels. Two types of forecast are foreseeable: 1) short term, in which catchment hydrology is modeled in real time at a fine resolution (e.g., 10-m to 30-m cells) and areas with probable high concentrations of developing larvae are identified and targeted for control; 2) long term, in which the impact of 6- to 12-month regional climate forecasts on local mosquito abundance is assessed by a coupling of climate forecast models and our hydrology model. The former system would require only the input of weather data from the nearest meteorologic station. Such monitoring could be centralized and carried out for large regions.

Mr. Shaman is a doctoral candidate in the Department of Earth and Environmental Sciences at Columbia University, New York City. His research interests include climatology, hydrology, vector-borne diseases, and mosquito ecology.

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Address for correspondence: Jeffrey Shaman, Lamont Doherty Earth Observatory, Columbia University, Oceanography, Room 106, Route 9W, Palisades, NY 10964, USA; fax: 845-365-8736; e-mail: jshaman@ldeo.columbia.edu

Surveillance for Antimicrobial Resistance in Croatia

Arjana Tambic Andraševic,* Tera Tambic,† Smilja Kalenic,‡ and Vera Jankovic,§ and the
Working Group of the Croatian Committee for Antibiotic Resistance Surveillance[1]

We describe the activities of the Croatian Committee for Antibiotic Resistance Surveillance and report surveillance results for 1999. Twenty-two Croatian microbiology laboratories participated in the study. Resistance rates for the organisms isolated in different centers varied widely, but certain trends were apparent. Penicillin resistance in pneumococci (38%), methicillin resistance in *Staphylococcus aureus* (22%), the production of extended spectrum beta-lactamases by *Klebsiella pneumoniae* (21%), and imipenem resistance in *Pseudomonas aeruginosa* (11%) represent major resistance problems, especially in large hospitals. A comprehensive system of antimicrobial resistance surveillance, combined with training and external quality control programs, has identified high rates of resistance in key pathogens in some regions of Croatia. The program has heightened awareness of the problems of antimicrobial resistance and contributed to ongoing improvements in laboratory practice.

A ntimicrobial resistance is a well-recognized problem worldwide (1). Resistant organisms are more likely in intensive care settings (2), where a combination of debilitated patients, invasive technology, and high antimicrobial use facilitates infections by multidrug-resistant staphylococci, enterobacteria resistant to third-generation cephalosporins, and imipenem-resistant nonfermentative bacteria. However, resistance is also a growing problem in community-acquired infections. Of particular concern are penicillin-resistant pneumococci (3-5) and the extended-spectrum beta-lactamase-producing enterobacteria (6). In addition, the World Health Organization (WHO) and the International Union against Tuberculosis and Lung Disease have shown that resistant tuberculosis (TB) is a problem in many parts of the world (7). Antimicrobial resistance often leads to therapeutic failure of empirical therapy; therefore, knowledge of the local prevalence of pathogens and their antimicrobial sensitivity patterns is essential for clinicians in their routine work. Clinicians should also be aware of the sensitivity patterns in both neighboring and distant areas.

In 1996, the Croatian Academy of Medical Sciences established the Committee for Antibiotic Resistance Surveillance in Croatia. The aims of the committee are to standardize methods for antimicrobial sensitivity testing in laboratories throughout the country, collect local data on antimicrobial resistance, and share the information with clinicians and pharmaceutical companies. The ultimate goal is a more appropriate use of antimicrobial agents in empirical therapy of infectious diseases. We present results of the surveillance for 1999 and describe the organization and activities of the committee.

Methods

Administration and Activities

Seventeen microbiology laboratories, representing the major geographic regions of the country, were initially asked to join the committee. Membership is now open to all 32 laboratories in Croatia. By 1999, 22 laboratories had joined the committee (Figure 1). The committee also includes infectious disease and clinical pharmacology specialists who are interested in antimicrobial resistance.

The committee meets twice a year to identify pathogens and antimicrobial agents to be surveyed in the next study period (June 1 to December 31). A smaller working group is set up to organize surveillance and produce forms for data collection. The forms are sent to the collaborating centers, then returned to the working group for analysis. The committee also organizes a focused study of one particular clinical problem (e.g., a study on methicillin-resistant *Staphylococcus aureus* [MRSA] and two studies of blood-culture isolates). Each year the committee summarizes its results in reports sent to the collaborating centers and made available publicly.

Educational Activities

The first Croatian Symposium on Antibiotic Resistance was organized in 1993. After the Committee for Antibiotic Resistance Surveillance was founded, it assumed responsibility for organizing these symposia every 3 years. The committee also organizes a biannual Workshop on Antibiotic Resistance at the Croatian Congress of Infectious Diseases. Each year, in collaboration with the local office of the Croatian Medical

*University Hospital for Infectious Diseases "Dr F. Mihaljeviæ," Zagreb, Croatia; †Croatian Academy of Medical Sciences, Zagreb, Croatia; ‡Zagreb Clinical Hospital Center, Zagreb, Croatia; and §Croatian National Institute of Public Health, Zagreb, Croatia

[1]Vlatka Janeš Poje, Vlasta Gilic, Zdenka Kotarski, Blaza Krakar, Nastja Kucišec Tepeš, Ivanka Lerotic, Biserka Matica, Danica Milanovic Martinovic, Marina Payerl-Pal, Mirna Petanovic, Marinka Piškoric, Volga Punda-Poljc, Ljubomira Radolovicc, Ivanka Ritterman, Antonija Sokal, Jasenka Škrlin, Nataša Šterk-Kuzmanovic, Nevenka Tkalec-Makovec, Dubravka Vukovic.

Figure 1. Croatian microbiology laboratories participating in surveillance of antimicrobial resistance.

Association, the committee organizes a 1-day meeting on antibiotic resistance in different counties. In addition, the committee organizes an annual course on laboratory methods that is mandatory and free of charge for the heads of the collaborating laboratories.

Quality Control

The Department of Microbiology at the University Hospital for Infectious Diseases in Zagreb participates in the WHO and Centers for Disease Control and Prevention External Quality Assurance program and acts as Croatian coordinator for member laboratories. This is the only organized quality assurance program in Croatia, and all committee members are required to participate. The laboratories are also encouraged to use the WHONET program for internal quality control.

Surveillance and Laboratory Methods

The surveillance period was from June 1 to December 31, 1999. Organisms selected for surveillance included group A *streptococcus*, *Streptococcus pneumoniae, Staphylococcus aureus, Enterococcus* spp., *Escherichia coli, Klebsiella* spp., *Proteus* spp., *Enterobacter* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa, Salmonella* spp., and *Shigella* spp. Laboratories were asked to record all nonduplicate isolates of these species during the surveillance period and their sensitivities to selected antimicrobial agents. The information was recorded on paper forms and returned to the working group. The isolates were collected from all body sites, and surveillance of routine data did not include differentiation of colonization from infection. Data for *Mycobacterium tuberculosis* were adapted from the annual report of the Croatian Reference Laboratory for Tuberculosis, which includes data from all laboratories in the country that process specimens for TB.

Identification of Bacteria

All organisms were assessed by colony morphology and Gram stain. All the laboratories used the DNA-base and slide coagulase tests for identification of *S. aureus*, bacitracin disk for group A *Streptococcus*, and optochin disk for *S. pneumoniae*. Enterobacteria and *P. aeruginosa* were identified by different methods in different laboratories.

Antimicrobial Sensitivity Testing

All participating laboratories used the disk diffusion method of the National Committee for Clinical Laboratory Standards for antimicrobial sensitivity testing (8). Disks for the selected antimicrobial agents were provided by the committee to ensure that all isolates were tested for all agents requested.

Results

The centers providing data included 22 hospital and public health laboratories (Figure 1). Resistance rates for the organisms isolated in different centers varied widely, but certain trends were apparent.

Gram-Positive Bacteria (Figure 2)

Among group A *Streptococcus* isolates, the overall resistance was 19% to erythromycin and 6% to clindamycin. *S. pneumoniae* isolates included those from primarily sterile sites, as well as from respiratory specimens and nasopharyngeal swabs. Resistance to penicillin and other antimicrobial agents was common. Both penicillin and cotrimoxazole resistance averaged 38%, macrolide resistance 23%, and tetracycline resistance 21%. Chloramphenicol resistance was relatively uncommon, averaging 7%.

These figures, however, obscure a wide range of results from different centers. Penicillin resistance ranged from 8% in Osijek to 62% in Cakovec; erythromycin and azythromicin resistance from 9% in Dubrovnik to 31% in one Zagreb center; and cotrimoxazole resistance from 14% in Sisak to 70% in Pula.

Overall, 18% of Croatian isolates of *S. aureus* were resistant to oxacillin (and therefore methicillin). Methicillin-resistant strains were often multidrug resistant, averaging 65% resistance to gentamicin, 49% to clindamycin, 57% to ciprofloxacin, and 50% to rifampicin. Resistance to fucidic acid (5%), cotrimoxazole (7%), and mupirocin (7% when tested by a 5-mg disk) was uncommon. Again, wide variation in resistance rates was seen, with strikingly low rates of MRSA in the Cakovec General Hospital.

Overall, only 2% of *Enterococcus* spp. isolates were resistant to ampicillin and 1% to nitrofurantoin. Resistance to gentamicin was 12% (when tested by a 120-mg disk), and vancomycin-resistant isolates occurred in only one Zagreb hospital (with 4% resistance).

Gram-Negative Bacteria (Figure 3)

Approximately half the *E. coli* isolates were resistant to ampicillin, and 15% were resistant to beta-lactam/beta-lacta-

Figure 2. Resistance rates (%) to selected antimicrobial agents in gram-positive bacteria in Croatia (June 1 to December 31, 1999). The number of resistant organisms / number of organisms tested is given in brackets. Note: macrolide resistance = resistance to erythromycin and azithromycin; penicillin resistance in pneumococci = nonsusceptibility to penicillin; gentamicin resistance in enterococci = high-level resistance.

mase inhibitor combinations. Approximately 24% of isolates were resistant to cotrimoxazole, 13% to cefuroxime, 4% to ceftazidime, 7% to gentamicin, 5% to ciprofloxacin, and none to imipenem. In almost all centers, resistance to ampicillin was >40% and to cotrimoxazole >20%. In one Zagreb hospital, resistance to ceftazidime reached 15%, but in most centers it was <4%.

Klebsiella organisms showed a high degree of multidrug resistance: 34% were resistant to co-amoxiclav or ampicillin+sulbactam, 33% to cefuroxime, and 21% to both ceftazidime and gentamicin. These organisms also showed moderate resistance (8% to 10%) to the other clinically available aminoglycosides (netilmicin and amikacin) but were generally sensitive to ciprofloxacin (6% resistance). No imipenem resistance was recorded. However, resistance rates varied widely, with ceftazidime resistance ranging from 2% to 39%, gentamicin resistance from 2% to 44%, and ciprofloxacin resistance from 1% to 17%.

Croatian isolates of *P. aeruginosa* also showed high rates of resistance and multidrug resistance, again with variation among centers. The overall rates of resistance (and ranges for different centers) were piperacillin 22% (7% to 57%), ceftazidime 7% (2% to 20%), imipenem 11% (0% to 21%), gentamicin 44% (20% to

69%), and ciprofloxacin 33% (16% to 60%).

Salmonellas generally showed good sensitivity to all antimicrobial agents except ampicillin (19% resistant) and chloramphenicol (6% resistant). Shigellas were highly resistant to ampicillin (87%), tetracycline (79%), and cotrimoxazole (89%). Resistance to other antimicrobial agents was <10%.

There was no imipenem resistance in enterobacteriaceae, but in *Acinetobacter* spp. it was 1% (0% to 8%). A high percentage (53% to 88%) of *Acinetobacter* isolates were resistant to many antimicrobial agents, except to the combination ampicillin + sulbactam (18% resistance), amikacin (25% resistance), and netilmicin (26% resistance). *Proteus* spp. isolates generally showed good sensitivity to antimicrobial agents except to ampicillin (49% resistance) and cotrimoxazole (28% resistance). *Enterobacter* spp. were often multidrug resistant, with stable resistance to ceftazidime of 31%.

In 1999, 5,664 isolates of *M. tuberculosis* were recovered in 17 laboratories; 316 (5.9%) of them were resistant to one of the first-line antituberculosis drugs (streptomycin, izoniazid,
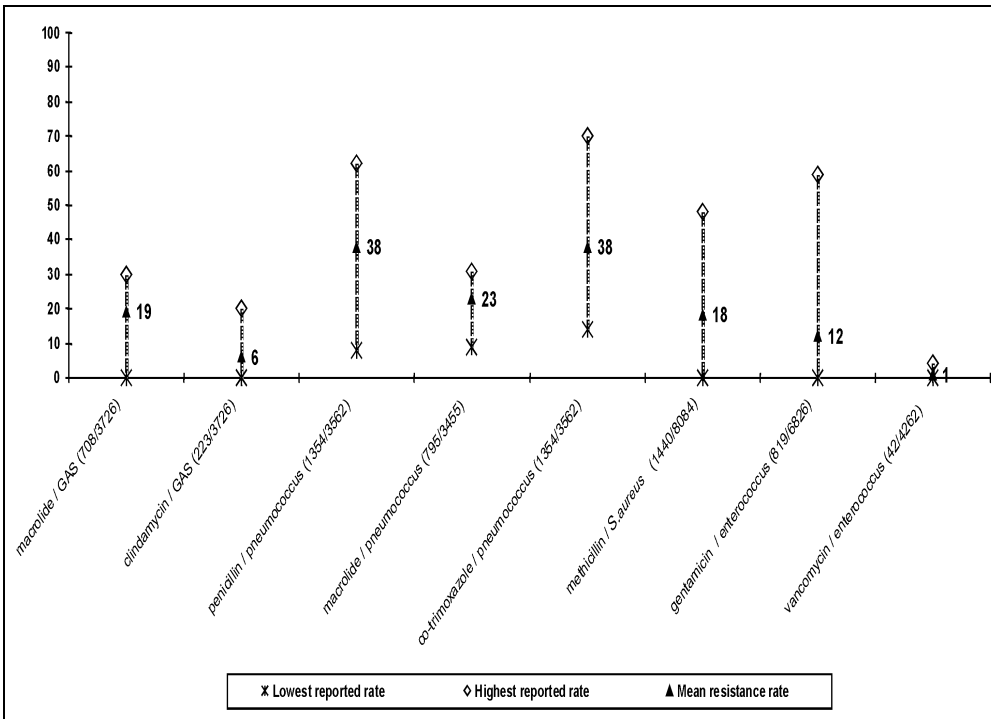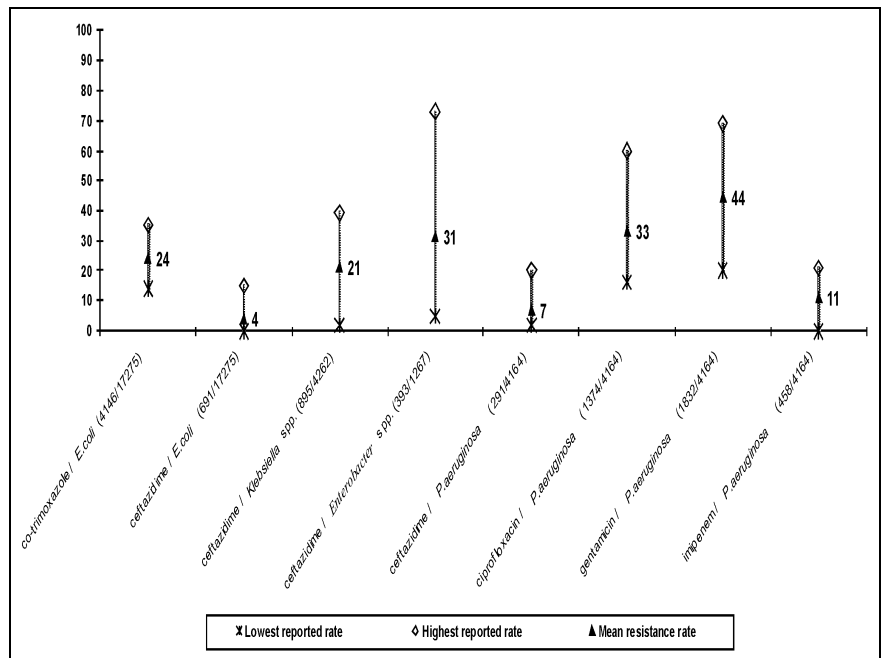

Figure 3. Resistance rates (%) to selected antimicrobial agents in gram-negative bacteria in Croatia (June 1 – December 31, 1999). The number of resistant organisms/number of organisms tested is given in brackets.

rifampin, pyrazinamid, or ethambutol). However, among newly diagnosed cases of TB, only 3% of isolates were resistant to one of the first-line drugs and 0.6% of isolates were multidrug resistant, i.e., resistant to rifampicin plus isoniazid.

Discussion

Croatian national data suggest that resistance is occurring in both community and hospital-acquired infections. *S. pneumoniae* is a major community-acquired pathogen. Resistance to penicillin of 38% has prompted an ongoing centralized study, which could also provide an estimate of the proportion of highly resistant strains.

MRSA is common, especially in large hospitals and on trauma wards. The incidence of MRSA increased rapidly during the early 1990s, frequently causing chronic osteomyelitis after war injuries. Observations in a trauma hospital in Zagreb suggest that MRSA spread throughout Croatia is facilitated by lack of screening and isolation facilities and poor interhospital communication (9,10). Some areas, such as the Medimurje region (city of Cakovec), are still almost unaffected by MRSA, and screening of all surgery and intensive-care unit patients transferred there from other centers is highly recommended.

Croatia does not seem to have a nationwide problem with vancomycin-resistant enterococci. Such isolates appear to be limited to the Clinical Hospital Center Zagreb, the largest hospital in Zagreb (11). Cotrimoxazole is still widely used in Croatia as the first-line antimicrobial agent for urinary tract infections. In many centers, resistance of *E. coli* and other enterobacteriaceae to this agent exceeds 20%, which indicates the need for alternative therapy for uncomplicated urinary tract infections. Production of extended spectrum beta-lactamases (ESBL) in *E. coli* is still rare, except in the Clinical Hospital Center Rebro in Zagreb. The first outbreaks of ESBL-producing *Klebsiella* organisms were described in Europe in the mid-1980s (12), and by 1999 >30% of *Klebsiella* isolates from three of five Zagreb hospitals were resistant to third-generation cephalosporins. It is already common for Croatian isolates of *P. aeruginosa* to be resistant to aminoglycosides, but of new concern is the finding that resistance to imipenem reaches 20% in some centers. While shigellas are usually multidrug resistant, salmonellas generally show good sensitivity to antimicrobial agents, except to ampicillin. ESBL-producing salmonellas, described as causing outbreaks both in the community and hospitals (6,13,14), were identified in Croatia for the first time in 2000 (unpub. data).

Anti-TB drug resistance is a particular problem throughout the world, with multidrug-resistant TB in new cases reaching >2% in one third of all countries (7,15). With 0.6% of multidrug resistance in new cases, Croatia has a low incidence of multidrug-resistant TB. However, difficulties with isolating patients and fully implementing the directly observed treatment strategy (16) complicate the situation.

Apart from providing national data, setting up a surveillance program has heightened awareness of the problem of antimicrobial resistance throughout Croatia. Both local and national data are published in the committee's annual report and distributed to the participating institutions. Such data are also discussed at a series of local meetings with clinicians and used as the basis for local antibiotic policies. The results of this nationwide surveillance serve as an early warning system for the emergence of antimicrobial resistance and indicate where more focused studies are needed.

The first year's experience showed that educational and external quality control programs were needed to supplement the surveillance program. Training courses organized by the committee improved the standardization of the laboratory procedures throughout the country and had great impact on detection of resistance mechanisms and infection control. By taking part in the surveillance network, microbiologists were stimulated to discuss antimicrobial resistance and treatment with ward staff, pharmacists, and managers; sensitivity testing methods were reviewed; and technical staff were taught to flag the isolation of resistant bacteria. This process is ongoing.

Laboratory methods need to be kept under continual review, and introducing a national quality control scheme was very helpful. An ongoing surveillance program would be greatly facilitated by the use of microcomputers connected by e-mail for constructing and updating electronic databases. The committee also intends to initiate collaboration with the Croatian Ministry of Health to ensure that official policies favor appropriate use of antimicrobial therapy.

Dr. Tambic Andrašević is a clinical microbiology consultant at the University Hospital for Infectious Diseases in Zagreb. Her primary interest is bacterial resistance to antibiotics, including surveillance and research on mechanisms of resistance.

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Address for correspondence: Arjana Tambic Andrašević, Department of Clinical Microbiology, University Hospital for Infectious Diseases "Dr. F. Mihaljevic," Mirogojska 8, 10000 Zagreb, Croatia; fax: 3851-467-8235; e-mail: arjana.andrasevic@zg.tel.hr

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

Adventure Health Conference

Navy Pier Conference Center

Chicago, Illinois
February 21-24, 2002

Health Issues in Adventure Travel is the focus of a new education day during the 12th Annual International Adventure Travel Show (IATOS). This seminar is cosponsored by the Centers for Disease Control and Prevention, the Wilderness Medical Society, Travel Health Resource, and the International Society of Travel Medicine. Session topics include The Increased Vulnerability of the Adventure Traveler, Unusual and Exotic Diseases of Adventure Travel, Minimizing the Risk of a Medical Crisis During International Travel, and Travel Wellness: How To Stay Fit and Health on the Road. In addition, the Division of Global Migration and Quarantine, National Center for Infectious Diseases, will present information from Geosentinel, a global surveillance network of the International Society of Travel Medicine and CDC, which monitors infectious diseases among mobile populations such as international travelers, immigrants and refugees.

For more information, contact Jennifer Gordon at jgordon@msemgmt.com or visit the conference website at www.IATOS.com .

The Dioxin Crisis as Experiment To Determine Poultry-Related *Campylobacter* Enteritis

Akke Vellinga and Frank Van Loock

In June 1999, the dioxin crisis, caused by dioxin-contaminated feed components, exploded in Belgium, resulting in withdrawal of chicken and eggs from the market. Through the sentinel surveillance system, a decrease in *Campylobacter* infections during June 1999 was noticed. A model was generated with the reports from preceding years (1994 to 1998), and a prediction of the number of infections in 1999 was calculated. The model shows a significant decline (40%) in the number of infections, mainly because of the withdrawal of poultry. The use of a disaster as an epidemiologic tool offers a unique opportunity to observe exceptional changes in the occurrence of infections or other diseases.

I n 1999, Belgium had a dioxin crisis caused by dioxin-contaminated feed being fed to livestock (1,2). The problem started at the end of January, when contaminated feed was processed; however, official notification of the crisis did not occur until the end of May (3). The source of the contamination was a fat-rendering company, where transformer oil with high levels of polychlorinated biphenyls (PCBs) and dioxins was used to manufacture animal foods. On May 28 (week 21), Belgian authorities ordered the withdrawal from sale of Belgian poultry and eggs; other European countries and Russia followed suit. On June 2, the European Community widened the ban and ordered the destruction of all food items containing >2% egg product and food containing chicken produced from January 15 to June 1 from infected farms (3). On June 4, 1999, the Belgian government issued a commerce embargo of meat products (pork and beef) with a minimum of 25% fat content, not applicable for dairy products. Meat was not withdrawn from sale.

In Belgium, a surveillance system of sentinel (n=127) and reference (n=38) laboratories, set up in 1983, reports on a voluntary basis on a list of organisms, including *Campylobacter*. Of all recognized private and hospital laboratories, 46% contribute to the surveillance system, which covers 35 of Belgium's 43 districts (4).

The number of registered *Campylobacter* infections increased from 2,534 cases in 1985 to 6,610 cases in 1998 and 6,521 in 1999. In Belgium, *Campylobacter* enteritis (campylobacteriosis) is mainly caused by *Campylobacter jejuni* (80% of the isolates) and *C. coli* (12%) (4).

Campylobacteriosis is a common form of infective diarrhea in industrialized countries; most infections are sporadic, and 80% are believed to be foodborne (5,6). *Campylobacter* can be isolated from many species of wild and domesticated animals, which are mainly asymptomatic carriers. Of farm animals, poultry and pigs are most frequently infected (5,7). Various risk factors have been suggested on the basis of case-control studies and outbreak investigations, including handling

Institute of Public Health–Louis Pasteur, Brussels, Belgium

chicken (8-10); eating not fully cooked chicken (11-13); eating commercially prepared chicken (13,14), sausages (10), and barbecue (10,15,16); exposure to farm or domesticated animals (10-13); or consumption of raw milk (12,13,16).

The dioxin crisis had implications for public health on more levels than the direct health effects of dioxin (17). Whereas the level of dioxin contamination and the health outcomes require research projects over long periods, one of the direct effects of this crisis could be noticed in the number of *Campylobacter* infections. This unique event made it possible to investigate the withdrawal of particular food products from the market and their contribution to campylobacteriosis.

Methods

To estimate the effect of the dioxin crisis on the number of *Campylobacter* infections, a model was designed by which the number of expected cases for 1999 could be calculated. This model was based on the data collected by the sentinel laboratory surveillance network from 1994 to 1998. The cumulative numbers per week were used to drive a monthly chronologic series for 1994 to 1998 and modeled according to the Fourier transformation (18,19). This procedure explains the cyclic patterns of data by spectral analysis; a complex time series with cyclic components is decomposed into sine and cosine terms describing the seasonal changes and a linear term to identify the trend (Figure 1).

The final model has three cyclical terms (52, 26, and 13 weeks), i.e., a strong yearly variation and two harmonics at the half and quarter year.

The linear trend shows a yearly increase from 1994 to 1998, with a slope of 15%. When the cyclical contributions are included, $r^2=0.86$, that is, 86% of the variation in the number of the *Campylobacter* infections can be explained by the model. The epidemic threshold is set at a distance of 1.96 standard deviations (SD); (95% confidence interval [CI]), which has shown to be useful for distinguishing epidemic increases from random variation (18), or (as for the dioxin crisis) exceptional, epidemic decrease. Outbreaks over the years were
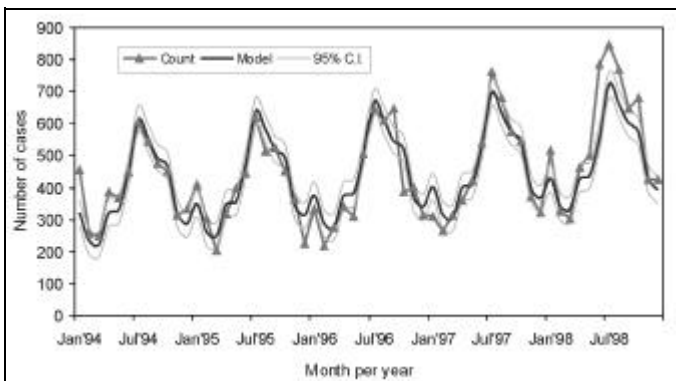
Figure 1. Campylobacteriosis in Belgium from 1994 through 1998, fit of the model.

smoothed for this calculation. Figure 2 shows the model, including the 95% CI and the actual numbers by which it was calculated. Poultry production per workday for 1998 and 1999 is also shown, representing the production per workday in indices with 1995 as reference year (1995 = 100) (20).

Age and gender distribution during the crisis was compared with the monthly distribution of age and gender in previous years. The model was made with MS Excel 2000; SPSS version 9.0 was used to compare distributions.

Results

Figure 2 shows the model, including the 95% CI as calculated from the numbers in the previous years during a period of 20 weeks (middle of April to the end of August, weeks 15 to 35) as well as the actual count for this period in 1999. The number of *Campylobacter* infections analyzed by the sentinel laboratory surveillance network fit the 95% CI except during the dioxin crisis (from week 21), when all poultry and eggs were taken off the supermarket shelves. The number of *Campylobacter* infections from week 23 to week 26 in 1999 is on average 94 cases per week or almost 40% lower than the
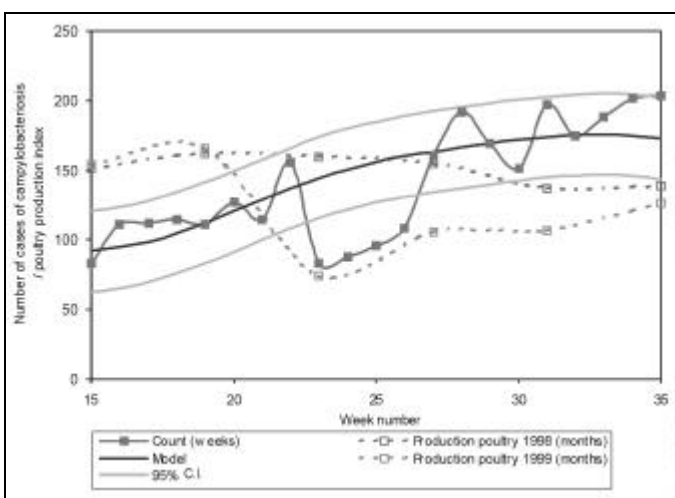
Figure 2. Campylobacteriosis in Belgium at period of dioxin crisis, model 1994–1998 and poultry production index.

expected average of 153 cases per week (SD_{model}=15 cases/week; SD = standard deviation). Overall, for the month of June (week 23 to 26), the expected number of infections was 643 (ST_{model}=61 cases/month) while the actual number of cases in 1999 was 375. The monthly calculated numbers follow the same trend as *Campylobacter* infections in 1999 and a similar decrease in numbers during the dioxin crisis. After 4 weeks, the ban was lifted, and the number of *Campylobacter* infections returned to the interval calculated by the model. Poultry production, even though over a longer period, also returned to levels comparable with the month of the previous year (1998). There was no difference in age or gender distribution of *Campylobacter* infections during the crisis compared with the rest of the year.

Discussion

The impact of the dioxin crisis can be detected in various disciplines. The economic impact is probably the easiest to determine, as this aspect is data driven. However, the dioxin crisis had a tremendous impact on health and health-related matters, including food consumption. The sudden change in food consumption, related to the withdrawal of poultry and eggs, had an immediate effect on the number of foodborne diseases, e.g., *Campylobacter*. The fitted model shows an unexpected decline in June 1999. Even though the decline in numbers is not exceptional, the moment at which this happens is. Looking at other declines in the number of *Campylobacter* infections, as in February 1996 or February 1997, these happen during a downward trend in numbers, while the drop in June 1999 occurs when numbers would normally be increasing.

The decline in campylobacteriosis and the lack of poultry in shops lasted 4 weeks, exactly the period from seizing all Belgian chicken and egg products from the supermarket shelves until the return of these items. This supports a direct link and contradicts a possible "ecological fallacy" as the time frame, which has an abrupt beginning and end, is similar for both the dioxin crisis and the decline in number of *Campylobacter* infections (21). During this period no other events possibly explaining the decline in numbers are known to have occurred. A major concern with ecologic studies is often the flue line (generally, the geographic boundaries of the occurrence of the risk factor and the occurrence of the illness), as was the case in the European study of the association between olive oil and cancer (22). The dioxin crisis differs from the commonly analyzed ecologic studies in this geographic aspect, since the borders of the impact of the dioxin crisis are the same as the borders of a well-established surveillance system.

Campylobacter is associated with several risk factors and risk behaviors, such as contact with farm and domesticated animals (mainly cats and kittens) (10-12) or recent history of antibiotic use (14). However, the dioxin crisis would not have an immediate effect on these factors, as it was primarily a food scare. Drinking raw milk, occasionally found to be a risk factor (12,16), is also unlikely to have contributed to the decline in numbers since milk products were not taken off the shelves.

Meat, in particular pork prepared on the barbecue, is generally accepted to be an important risk factor (10,16). Pork meat is associated with *C. coli*, which accounted for 12% (in 1998) and 21% (in 1999) of all *Campylobacter* infections (4). Meat remained available during the crisis, which might explain the small increase in *C. coli* infections, as it is imaginable that chicken, which is the main source of *C. jejuni* infections, was replaced by pork during the crisis.

Eggs were not on sale during the crisis, but they are generally not associated with *Campylobacter* contamination. (A possible link of eggs with *Salmonella* infections will be examined.)

The withdrawal of chicken and all related products from the supermarket during the dioxin crisis is the most likely reason for the sudden decline in *Campylobacter* infections. Chicken is found to be the principal source of infection in most case-control studies (11,13,14,16). In Seattle, rare, raw, and cooked chicken were all significantly associated with *Campylobacter* infection (8). Undercooked chicken was found to be a risk factor in a Colorado study (12). Handling raw and even frozen chicken, possibly because of cross-contamination in the kitchen, has also been significantly associated with campylobacteriosis (10). As all Belgian poultry was withdrawn, our data allow an estimation of the number of *Campylobacter* infections directly related to poultry.

The decline in the number of *Campylobacter* infections in Belgium by 40% was due to the withdrawal of Belgian poultry from the market. In 1999, 199,251 tons of poultry meat was available for human consumption; 81,261 tons (41%) was imported (23). Foreign poultry remained available on the market.

According to a marketing bureau that investigates trends in shopping behavior of 3,000 families in Belgium, eating habits have changed little because of the dioxin crisis (24); moreover, the overall purchase of poultry in 1999 increased by almost 9%. However, a shift was seen in the quality of the poultry sold; after the crisis, consumers preferred chicken with some sort of quality label, even though the current labels do not specifically address contamination issues.

Besides the 40% decrease in *Campylobacter* infections during the dioxin crisis, this experiment also highlights the remaining baseline of 60%, averaging 75 infections a month. According to an analysis of foodborne disease information in the United States (1999), only 80% of the *Campylobacter* spp. infections are estimated to be foodborne (5). Furthermore, as only Belgian chicken was banned and non-Belgian poultry was still on sale, a number of poultry-related infections are still present in the reported numbers. With at least 40% of the *Campylobacter* infections in Belgium explained by poultry and 20% by non-foodborne causes, the source of the remaining infections should be further explored and investigated.

The use of a disaster as an epidemiologic tool offers a unique opportunity to observe exceptional changes in the occurrence of infections or other diseases. The causes or consequences of the crisis can serve as treatment in an uncontrolled natural experiment. The dioxin crisis as experiment showed that >40% of the *Campylobacter* infections can be attributed to poultry.

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Akke Vellinga, University of Antwerp (UIA), Department of Epidemiology and Social Medicine, 2610 Antwerp, Belgium; fax: 323-820-4026; e-mail: akke.vellinga@ua.ac.be

Enhancing Public Health Surveillance for Influenza Virus by Incorporating Newly Available Rapid Diagnostic Tests

Paul V. Effler, Man-Cheng Ieong, Tammy Tom, and Michele Nakata

Beginning with the 1999-2000 influenza season, physicians throughout Hawaii ordering a viral culture for patients with suspected influenza were also offered influenza rapid testing. We compared the number of viral respiratory cultures sent to the Hawaii Department of Health and the number of providers who participated in influenza surveillance over consecutive influenza seasons. The number of viral respiratory cultures rose from 396 to 2,169 between the 1998-1999 and 2000-2001 influenza seasons, and the number of providers submitting ≥1 influenza culture increased from 34 to 327, respectively. The number of influenza isolates obtained each season also increased (from 64 to 491). The available data suggest that the changes observed in Hawaii's influenza surveillance were not secondary to differences in influenza activity between seasons. This is the first evaluation of integrating influenza rapid testing into public health surveillance. Coupling rapid tests with cultures appears to be an effective means of improving influenza surveillance.

"The importance of surveillance to the detection and control of emerging microbial threats cannot be overemphasized."

Institute of Medicine (1)

The recent, highly fatal, outbreak of human illness caused by a novel influenza A virus H5N1 in Hong Kong has dramatically underscored the importance of monitoring influenza activity in the United States as a part of national preparedness for a pandemic (2,3). Ongoing, comprehensive surveillance is vital to addressing influenza because influenza A viruses have the capacity to undergo abrupt shifts in the major antigenic determinants of their surface proteins (4); these shifts can give rise to novel influenza viruses capable of producing catastrophic pandemics (5). In 1918 an estimated 20 million people died from a new influenza A H1N1 virus strain (6). Milder influenza pandemics in 1957 and 1968 killed an estimated 90,000 people in the United States (1).

Enhancing our capacity to detect novel viruses with pandemic potential requires establishing comprehensive programs to culture and subtype influenza isolates in circulation during outbreaks and annual epidemics, in other words, virologic surveillance. With this goal in mind, the Hawaii State Department of Health (HDOH) sent letters in the fall of 1998 to all licensed physicians in the state encouraging them to collect specimens for viral culture when evaluating patients suspected of having influenza. Although these cultures were offered at no cost to the physician or patient, little increase in specimen submissions was observed during the 1998-99 influenza season when compared to prior years. Subsequent discussions with providers indicated that there was little incentive to collect viral cul-

ture specimens because culture results typically take 2 to 8 weeks to be finalized and thus are not perceived as useful for clinical management.

A more timely diagnosis of influenza is possible with rapid antigen tests (7). The advent of rapid testing for influenza has raised concern among public health professionals, however, because of the potential of such tests to undermine virologic surveillance. Some authorities have predicted that submissions of specimens for viral isolation would decrease as rapid antigen test kits are improved and become more widely available (3). The rapid tests currently available cannot distinguish novel virus subtypes from subtypes already known to be in circulation. If fewer viral isolates are obtained as a consequence of adopting point-of-care rapid tests, the result may be a reduction of our capacity for early detection of the next influenza pandemic.

We present an evaluation of the impact of incorporating rapid influenza testing into ongoing virologic surveillance activities.

Methods

Standard Virologic Surveillance

The virologic influenza surveillance system in Hawaii before the 1999-2000 influenza season consisted of physicians submitting cultures for influenza directly to HDOH (direct submissions). HDOH supplied the specimen collection materials, transported specimens, and processed the specimens without charge. There were no restrictions on the number of specimens that could be submitted. This service had been available for many years but was actively promoted through a mail-out in 1998 addressed to physicians throughout the state. Commercial clinical laboratories in Hawaii do not perform

influenza viral cultures locally, and very few specimens are forwarded from these laboratories to reference laboratories on the U.S. mainland (the number of influenza cultures sent out of state has not exceeded 25 specimens for any of the major laboratories during each of the last four influenza seasons [F. Liu, X. Zheng, pers. comm.])

Enhanced Virologic Influenza Surveillance for 1999-2000 and 2000-01

Beginning with the 1999-2000 influenza season, and again during the 2000-01 season, all licensed physicians in the state were informed by mail that rapid influenza tests were available to clinicians who also wished to order an influenza culture for their patient. The letter recommended considering the diagnosis of influenza in patients with a fever ≥37.8°C accompanied by a cough or sore throat.

HDOH supplied viral specimen collection kits and rapid test kits to four private clinical laboratories that provide service to approximately 80% of the state's population, at no charge to the laboratory (8). The specimen collection kits contained two sterile dacron swabs, one sterile polystyrene tube, one tube containing viral transport medium (VTM Micro Test, Inc.; Lilburn, GA), and instructions for collection and submission of the appropriate specimens. Participating commercial laboratories were responsible for distributing these supplies to their satellite specimen collection locations (>70 distinct locations overall). To obtain both a rapid influenza test and viral culture, two throat or nasopharyngeal swabs were required, one placed into the sterile polystyrene tube and the other into the tube containing VTM. Specimens were transported through usual specimen transport channels.

Rapid influenza tests were performed at participating commercial laboratories, and the results were reported to the submitting provider and HDOH. Laboratories were not allowed to bill for the cost of rapid test kits provided by HDOH but could charge for performing the test per their usual practices. The specimen in VTM was then forwarded from the commercial laboratory to the Hawaii Public Health Virology Laboratory (HPHVL, a World Health Organization [WHO] collaborating virology laboratory) for culture, typing, subtyping, and antigenic characterization.

HDOH purchased the influenza rapid tests through a competitive bid process. The primary criteria used for selecting the BioStar FLU OIA test (Boulder, CO) was cost (<U.S.$5 for the first 1,000 tests and <$10 for any additional tests). The test manufacturer provided training on performing the rapid test to HPHVL and commercial laboratory staff.

For this report, the number and results of influenza cultures submitted and the number of distinct providers who submitted at least one viral culture during the 1997-98, 1998-99, 1999-2000, and 2000-01 influenza seasons were determined. The influenza season was defined as the period beginning with week 40 of the first year extending through week 20 of the next year, that is, early October through mid-May. In addition, for the 2000-01 influenza season, we calculated the positive and

negative predictive values of the FLU OIA Rapid Test as compared to influenza viral culture for all specimens with complete test results as of May 21, 2001.

Comparison Data

After observing a change in the number of viral cultures submitted between the 1999-2000 influenza season and the previous season, we conducted an assessment to determine if this difference might be explained by factors other than incorporation of rapid tests into the surveillance effort. Data regarding the number of influenza cultures performed at sites other than Hawaii for the 1997-98, 1998-99, and 1999-2000 influenza seasons were obtained from the Centers for Disease Control and Prevention (CDC) for approximately 75 other WHO collaborating virology laboratories located throughout the United States (9). Most of the participating laboratories are located in state or local health departments with a smaller number in universities or hospitals. During the 1999-2000 influenza season, 44 of the WHO laboratories (excluding Hawaii HPHVL) reported information for at least 25 weeks of the season; 37 (84%) of the 44 laboratories had also supplied reports for at least 25 weeks during each of the two prior influenza seasons, and these laboratories were included in our analysis.

Data from physician offices in Hawaii participating in the U.S. Influenza Sentinel Physician Surveillance Network (ISPSN) were used as a measure of the relative severity of Hawaii's past four influenza seasons (10,11). ISPSN sites report the total number of patient visits and the number of visits for influenza-like illness on a weekly basis during the influenza season. Influenza-like illness was defined as a fever of ≥37.8°C accompanied by a cough or sore throat. Weeks for which the proportion of visits for influenza-like illness exceeded 3% were defined as having increased influenza activity (10).

Results

The total number of specimens submitted to HPHVL for influenza culture increased sharply during the first season that rapid tests were introduced into influenza surveillance; this trend continued into the 2000-01 season (Table 1). The number of influenza isolates obtained tripled between the 1998-99 and 1999-2000 influenza seasons and then tripled again between the 1999-2000 and 2000-2001 seasons. A nearly 10-fold increase in the number of distinct providers who submitted influenza cultures was observed between the 1998-99 and 2000-01 influenza seasons.

A total of 1,015 (91%) and 2,101 (97%) of the culture specimens received at HPHVL were processed through one of the participating private laboratories during the 1999-2000 and 2000-01 influenza seasons, respectively; 2,979 (96%) of these culture specimens had a rapid test performed on an accompanying specimen; the remaining specimens were direct submissions to HPHVL. All four of the private laboratories materially participated in the surveillance activity; the mean number of specimens submitted by participating laboratories was 237,

Table 1. Number of influenza cultures submitted, isolates obtained, sub-typed and antigenically characterized, and the number of providers participating in surveillance, compiled by influenza season, Hawaii.

	Influenza season [a]			
Number of:	1997-98	1998-99	1999-2000[b]	2000-01[b]
Cultures submitted	306	396	1112	2169
Influenza isolates	73	44	137	491
Influenza A isolates	73	35	136	202
(Number subtyped)	(10)	(20)	(0)	(159)
Influenza B isolates	0	9	1	289
Providers submitting one or more cultures	25	34	196	327

[a] The influenza season was defined as the period beginning with week 40 of the first year extending through week 20 of the next year, i.e. early-October though mid-May.

[b] Rapid tests incorporated into influenza surveillance during this season.

with totals ranging from 188 to 333 during the 1999-2000 influenza season.

None of 136 influenza A isolates obtained during the 1999-2000 influenza season were subtyped at HPHVL. However, 159 (79%) of 202 influenza A isolates identified during the 2000-01 were subtyped and characterized at HPHVL; 143 were A/New Caledonia/20/99-like (H1N1), and 16 were A/Panama/2007/99-like (H3N2).

Influenza Cultures Submitted to WHO Collaborating Laboratories in the United States

A 5% increase was observed in the number of influenza cultures submitted between the 1998-99 and 1999-2000 influenza seasons for the 37 WHO comparison sites overall (Table 2). When data for each laboratory were examined individually, Hawaii HPHVL demonstrated the largest rise in influenza cultures processed between the 1998-99 and 1999-2000 seasons (181%); the next highest increase was reported from a laboratory in the Mountain region, whose specimens increased from 260 to 524 (a 102% increase) between the 1998-99 and 1999-2000 seasons (individual data for laboratories other than Hawaii are not shown). Three other laboratories had increases of approximately 50% over the previous influenza season, but more than half (19 of 37) of the laboratories had fewer specimens submitted in the 1999-2000 season when compared to the previous season.

Physician Visits for Influenza-Like Illness

The number and proportion of patients with influenza-like illness reported from sentinel physicians in Hawaii for the past four consecutive influenza seasons are shown in Table 3. The percent of patient visits for influenza-like illness during the 1999-2000 and 2000-01 influenza seasons was generally similar to that reported during the two prior seasons. The number of weeks that this proportion exceeded baseline were 21 for the 1997-98 and 1998-99 influenza seasons and 17 for the 1999-2000 and 2000-01 seasons.

Comparing the Influenza Rapid Test with Culture

A two-by-two table comparing the results of the FLU OIA test and culture results obtained during the 2000-01 influenza season is presented (Figure). In our setting, the positive predictive value of the FLU OIA was 51%, and the negative predictive value was 84%.

Conclusion

This is the first evaluation of the effects of integrating rapid testing for influenza into public health surveillance. Surveillance incorporating rapid tests greatly increased the number of specimens submitted for viral culture and the number of influenza isolates obtained. This finding is important because enhancing our capacity to recover circulating influenza virus isolates is the first step in establishing a robust virologic surveillance system designed to detect novel viral strains with pandemic potential.

Table 2. Number of influenza cultures performed at 37 World Health Organization collaborating virology laboratories located throughout the United States (excluding Hawaii), aggregated by region and compiled by influenza season[a]

	Influenza season			% Change from 1998-99 to 1999-2000
	1997-98	1998-99	1999-2000	
Hawaii	306	396	1,112	181%
Regional U.S. totals				
East South Central	190	339	424	25%
West North Central	7,225	7,218	8,663	20%
Mountain	4,456	5,531	6,471	17%
Mid-Atlantic	3,421	3,785	4,191	11%
Pacific (excl. Hawaii)	2,288	3,214	3,353	4%
South Atlantic	8,510	8,637	8,368	3%
West South Central	1,040	2,291	2,173	5%
New England	1,308	1,720	1,555	10%
East North Central	3,917	4,041	3,572	12%
Total for laboratories outside Hawaii	32,355	36,776	38,770	5%

[a] Data for the 2000-01 season were not yet available at the time this report was compiled.

Table 3. The number and proportion of visits for influenza-like illness (ILI) reported from Hawaii physicians participating in the U.S. Influenza Sentinel Physician Surveillance Network, by influenza season

Influenza season	Number of participating physicians	Number of visits for ILI [a]	Total number of visits	Overall % of visits for ILI	Number of weeks proportion of visits for ILI exceeded baseline [b]	Highest weekly proportion of visits for ILI
1997-98	11	805	25,085	3.2%	13	10%
1998-99	11	728	31,308	2.3%	8	6%
1999-2000	7	272	14,831	1.8%	6	8%
2000-01[c]	15	819	30,722	2.7%	11	6%

[a] ILI is defined as a fever of $\geq 37.8^{o}$ C accompanied by a cough or sore throat.

[b] ILI baseline is defined as < 3% (see reference 10).

[c] Data avaialble through May 16, 2001.

Although physicians were permitted to send specimens directly to HDOH for viral culture, most of the influenza culture specimens submitted during the 1999-2000 and 2000-01 seasons were collected concurrently with a specimen for rapid testing. The fact that so few culture specimens were collected without a companion specimen for rapid testing implies that many physicians felt there was value to having both a culture and rapid test result, or alternatively, that the rapid test served as an incentive to obtain a culture.

The dramatic increase in the number of physicians who participated in the enhanced influenza surveillance project as compared to standard virologic surveillance suggests that the availability of the rapid test may have appealed to a larger cohort of clinicians. The involvement of a greater number of physicians is a promising development because public health officials may ultimately be able to obtain a more extensive and representative sample of circulating influenza isolates by broadening the pool of patients sampled.

This assessment is limited in that it lacks a control group consisting of physicians and laboratories that did not have access to the free rapid influenza tests for comparison once the enhanced virologic influenza project was initiated, beginning with the 1999-2000 influenza season. Instead, we compared the number of specimens received during the 1999-2000 and

2000-01 influenza seasons to the number received in the two prior seasons. Because the severity of annual influenza seasons can vary greatly from year to year, it could be argued that the increased number of specimens submitted during the latter seasons was secondary to increased numbers of influenza-like illnesses during those seasons. Several sources of information suggest this was not the case, however. First, influenza-like illness surveillance in Hawaii over the 4-year period does not suggest that influenza activity was markedly increased during the last two seasons. Second, the number of influenza cultures performed at laboratories on the mainland during the 1999-2000 season did not increase over the previous season to the extent observed in Hawaii. Third, CDC's published assessment of the 1999-2000 influenza season was that the "season's activity was similar to the previous two" and influenza activity during the 2000-01 season was assessed as "moderate and lower than the previous three seasons" (12,13).

During the first season in which rapid tests were introduced, HDOH was unprepared for the increased numbers of submissions for influenza culture. HPHVL reported that the high volume of specimens received during the peak period placed an unexpected strain on virology section's resources and that shortages in host cells, incubator space, and laboratory supplies resulted in a delay of several weeks for processing approximately 100 specimens (S. Naka, pers. comm.). The increased workload from processing specimens at HPHVL greatly reduced the laboratory's resources for subtyping the influenza A isolates obtained that season. While recovering isolates is a critical first step in developing our capacity for influenza surveillance, to truly improve pandemic preparedness any increases in virus isolation must be coupled with expanded subtyping of influenza A isolates. Recognizing this, and with the experience gained from the prior year, adjustments in staffing and procurement permitted 79% of the influenza A isolates to be subtyped during the 2000-01 season.

Because of the difficulties encountered in processing specimens during the 1999-2000 influenza season, we limited our assessment of the influenza rapid test as compared to culture to specimens received in the 2000-01 season. This project was

	Positive	Negative	
Flu OIA Test Result Positive	195	188	383
Flu OIA Test Result Negative	278	1,408	1,686
	473	1,596	

Positive predictive value: (195/383) =   51%
Negative predictive value: (1,408/1,686) =   84%

Figure. Comparison of FLU OIA and viral culture results, 2000-01 influenza season, Hawaii.

not designed as an evaluation of the rapid influenza test: We did not control for patient selection, specimen collection, or laboratory technique. Therefore, the findings from this field evaluation may not represent the rapid test's performance in other settings. Other assessments on the performance of influenza rapid tests are available (5,7,14).

Finding that isolates recovered during the influenza season increased when we integrated influenza rapid testing into our surveillance system should help allay concerns that adopting these tests will undermine virologic surveillance. If rapid influenza tests become an established component of clinical management, it will be difficult to convince clinicians to collect culture specimens in lieu of performing rapid tests (7,15). Collection of a second specimen for culture confirmation, on the other hand, may be seen as clinically appropriate. Therefore, public health agencies may ultimately benefit from incorporating rapid diagnostic tests into influenza surveillance programs.

None of the commercial laboratories that participated in this project were independently offering rapid influenza testing before the 1999-2000 season. Through this initiative, each of the laboratories gained a level of proficiency in performing the rapid influenza test, and physicians became familiar with ordering the test. Once rapid influenza testing becomes established in laboratory and clinical practice, the question of whether providers will continue to opt for a rapid test and viral culture provided by a public health agency, or simply order a rapid test from a private laboratory at the patient's expense, is still unanswered.

Although Hawaii's Enhanced Influenza Virologic Surveillance program is a good model of a public-private partnership (16), we have one potential concern about the impact of using rapid influenza tests on public health surveillance. To date, at least two private influenza surveillance systems using rapid tests have been established–one by a manufacturer of a rapid test kit and the other by a pharmaceutical company that produces an anti-influenza medication (17,18). Large private disease surveillance systems are relatively new to medicine. Because the chief concern of these proprietary systems is likely to be increasing sales of a product, it is not clear what role they will play in protecting the public health. If private surveillance systems compete with public agencies for physician participation, they may adversely affect virologic influenza surveillance.

In summary, our findings demonstrate that rapid influenza tests can be successfully integrated into public health surveillance efforts, resulting in a larger number of influenza isolates being available for subtyping and antigenic characterization. This enhanced influenza surveillance effort was accomplished through mutually beneficial public-private partnerships with commercial laboratories that routinely provide service to community physicians and their patients. Preparing for the next influenza pandemic compels public health agencies to work with physicians to expand our capacity for influenza surveillance. As the available data indicate that recent influenza pandemic strains have originated in Asia, vigilant virologic surveillance is especially important for Hawaii (19). Should an anomalous influenza strain emerge from Asia again in the future, our state's unique geographic location and visitor profile make it likely that our population will be among the first in the United States to encounter this new pathogen.

Dr. Effler is the State Epidemiologist with the Hawaii State Department of Health. His research interests include infectious disease surveillance, electronic laboratory reporting, campylobacteriosis, and leptospirosis.

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Address for correspondence: Paul Effler, Department of Health, Room 444, 1250 Punchbowl Street, Honolulu, HI 96813, USA; fax 808-586-8347; e-mail: pveffler@mail.health.state.hi.us

Participant Blinding and Gastrointestinal Illness in a Randomized, Controlled Trial of an In-Home Drinking Water Intervention

John M. Colford, Jr.,*† Judy R. Rees,*† Timothy J. Wade,*† Asheena Khalakdina,*† Joan F. Hilton,‡ Isaac J. Ergas,* Susan Burns,* Anne Benker,* Catherine Ma,§ Cliff Bowen,§ Daniel C. Mills,§ Duc J. Vugia,§ Dennis D. Juranek,¶ and Deborah A. Levy¶

We conducted a randomized, triple-blinded home drinking water intervention trial to determine if a large study could be undertaken while successfully blinding participants. Households were randomized 50:50 to use externally identical active or sham treatment devices. We measured the effectiveness of blinding of participants by using a published blinding index in which values >0.5 indicate successful blinding. The principal health outcome measured was "highly credible gastrointestinal illness" (HCGI). Participants (n=236) from 77 households were successfully blinded to their treatment assignment. At the end of the study, the blinding index was 0.64 (95% confidence interval 0.51-0.78). There were 103 episodes of HCGI during 10,790 person-days at risk in the sham group and 82 episodes during 11,380 person-days at risk in the active treatment group. The incidence rate ratio of disease (adjusted for the clustered sampling) was 1.32 (95% CI 0.75, 2.33) and the attributable risk was 0.24 (95% CI -0.33, 0.57). These data confirm that participants can be successfully blinded to treatment group assignment during a randomized trial of an in-home drinking water intervention.

I n 1991, Payment and colleagues described a randomized, controlled intervention trial designed to evaluate whether the consumption of tap water treated conventionally to meet regulatory standards affects incidence of gastrointestinal (GI) illness (1). In this trial, reverse osmosis filters were installed in 299 households (1,206 persons), and another 307 households (1,202 persons) were followed as controls, with no device installed. After prospective follow-up over a 15-month period, the investigators concluded that 35% of the self-reported GI illness was attributable to tap water consumption. A second trial conducted several years later included treatment groups receiving regular tap water, tap water from a continuously purged tap; bottled treatment plant water; and purified bottled plant water (2). This second study attributed 14% to 40% of GI illness to consumption of tap water that met Canadian water treatment standards. Because participants in these studies were not blinded to their treatment group assignments, GI illness may have been overreported by subjects in the tap water groups.

In 1996, the Safe Drinking Water Act of 1974 (3) was amended to require the Centers for Disease Control and Prevention (CDC) and the Environmental Protection Agency (EPA) to provide a national estimate of waterborne infectious disease in the United States. In the late 1990s, these agencies funded a large, randomized trial to evaluate the risk for GI illness from the consumption of tap water treated to meet all federal drinking water standards. As a preliminary step in the determination of the national estimate, CDC and EPA funded this pilot study to determine the feasibility of water intervention trials blinding participants to group assignment.

We report the results of the Pilot Water Evaluation Trial (Pilot WET), a randomized, controlled, triple-blinded intervention trial performed in 1999 in households in Contra Costa County in northern California. The primary objective of the trial was to assess whether, for 4 months, participants could be successfully blinded to group assignment, a (sham or active) water treatment device installed underneath the kitchen sink. Secondary objectives included estimating rates of highly credible gastrointestinal illness (HCGI) and other health outcomes and determining the feasibility of performing a similar trial on a larger scale.

Methods

The study and the informed consent process were reviewed, approved, and monitored by six Institutional Review Boards (Human Subjects Protection Committees) from the investigators' institutions (University of California, Berkeley; the University of California, San Francisco; the California Department of Health Services; and Public Health Foundation Enterprises, Inc.) and the funding agencies (CDC and EPA).

*University of California Berkeley, Berkeley, California, USA; †California Emerging Infections Program, Berkeley, California, USA; ‡ University of California San Francisco, School of Medicine, San Francisco, California, USA; §California Department of Health Services, Berkeley, California, USA; and ¶Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Study Area, Water Supply and Water Distribution System

The study area included single-family dwellings served by the Contra Costa Water District. The treatment plant serving the study area used standard conventional treatment with chloramination. A new ozonation plant was completed during the study period, so that after May 1999 the water supply was also ozonated. Source water from the San Joaquin River delta contained agricultural and industrial runoff and pathogens, including *Cryptosporidium*. (More detailed water characterization for the district may be found at http://www.epa.gov/enviro/html/icr/utility/report/CA0710003961023144135.html). Nonetheless, the finished water meets all federal and state drinking water treatment standards and requirements.

Recruitment, Enrollment, and Compensation of Households

Households were recruited by the Survey Research Center at the University of California, Berkeley, through hand delivery of information packets describing the trial, and by telephone recruitment with a reverse directory in the targeted enrollment areas. To be eligible for the trial, families were required to own their homes, use municipal tap water as the principal drinking water source, and have no household members with a serious immunocompromising condition (such as HIV/AIDS or cancer). Households received $40 on enrollment and an additional $160 in installments on the return of completed health diaries throughout the trial. The first device was installed in March 1999 and the final device in October 1999. Each family was asked to participate for 16 weeks.

One member of each household, designated the "index respondent," was responsible for communications between the household and the Survey Research Center. The index respondent was the adult member of the household who was in the best position to complete health diaries for other household members who were unable to do so. For 16 weeks, the index respondent mailed completed questionnaires every 2 weeks to the Survey Research Center.

Randomization and Blinding

Two random sequences were generated to allocate households 50:50 to active or sham filtration devices in blocks of 20. Blocking ensured approximate balance in the number of households per device as participants accrued. One study investigator, who remained unblinded throughout the trial and had no role in data analyses, prepared coded labels from the sequences and sent them to the manufacturer; the manufacturer permanently affixed the labels to the devices. All other study investigators, the plumbing contractor who installed the devices, and the study subjects were blinded as to the household device assignment throughout the trial, including the analysis phase, resulting in a triple-blinded trial.

Statistical Methods: Blinding Index and Sample Size

The sample size requirement was based on the primary aim of the trial: to determine if subjects could be blinded to water filtration device type. The effectiveness of blinding was quantified by the Blinding Index (BI) of James et al. (4), which can be expressed as $BI = p \times (r/2) + q$, where p is the proportion who attempt to guess their device assignment, $0 \leq p \leq 1$; r is the ratio, among those who attempt guesses, of the proportions of observed and expected guesses; and $q = 1 - p$ is the proportion of subjects who do not attempt to guess (i.e., report they "don't know"). When $r/2 = 0$, $BI = q$, and when $r/2 = 1$, $BI = 1$; thus $0 \leq BI \leq 1$. If correct guesses are weighted by 0.0 as recommended (4), then $r/2$ depends only on incorrect guesses. If, in addition, the weights and expected proportions are equal for all incorrect guesses, the term $p \times (r/2)$ equals the proportion of incorrect guesses; otherwise, $p \times (r/2)$ approximates the proportion of incorrect guesses. Thus, BI can be the same or nearly the same as the sum of the proportions of incorrect and "don't know" responses. The expected values used in the ratio r are calculated under the hypothesis that assignments and guesses are independent (i.e., the subjects are blinded); under this hypothesis $r/2 = 0.5$, whereas when the observed proportion of incorrect guesses exceeds the expected proportion $r/2 > 0.5$. Assuming that both incorrect and "don't know" responses are consistent with blinding, James et al. (4) suggest that when the BI is >0.5 the subjects have been blinded successfully on average.

We designed the study to test the null hypothesis with a type I error rate of 0.05, a type II error rate of 0.10, and a variance estimated by $BI(1 - BI)$. In simulations, the distribution of the BI was found to be approximately binomial (data not shown), and this distribution was assumed for variance estimation when the necessary sample size was calculated. Assuming an average household size of 2.4 persons, on the basis of census data, and an intrahousehold correlation of 0.60, based on the work of Donner, Birkett, and Buck (5), 80 households, 40 per group, were required.

Active and Sham Water Treatment Devices

Devices for our trial were purchased from Freshwater Systems, Australia, and installed by Assured Water Products, Inc., a licensed plumbing firm based in Contra Costa County. The devices were designed to be externally identical and to differ only in their ability to remove microorganisms from water.

The active water treatment device contained a 1-micron absolute prefilter cartridge and a UV lamp secured in a quartz sleeve that permitted transmission of UV light. The lamp was designed to emit UV light at 254 nm (optimum for disinfection) with a total minimum dose of 38,000 μ watt-sec/cm^2 to reduce postfiltration bacteria and viruses by $\geq$99% (6). The manufacturer provided written certification that the lamp would emit UV light above this level for 1 year.

The sham device contained an empty filter housing and a UV lamp in a glass sleeve that prevented the transmission of UV light to the water. Inside the empty filter housing, a plastic tube was glued to the inlet to circulate incoming water throughout the empty housing tank to prevent stagnation. Both devices had a tamper-proof seal to prevent opening of the filter casing

and an alarm that would sound in the event of failure of the UV lamp or power supply. The devices, installed under the kitchen sink on the cold water line, included a separate drinking water tap at the sink. Both devices provided a water flow through the tap of 5 liters per minute. The cost of the water treatment device, including plumbing expenses, was approximately $988 per household.

Blinding Outcomes

Every 2 weeks, participants aged $\geq$12 years were asked to report on a questionnaire one of five possible responses: "It is definitely the active water treatment device;" "It is probably the active water treatment device;" "It is probably not the active water treatment device;" "It is definitely not the active water treatment device;" or "I'm not sure." To accommodate the blinding index, these responses were collapsed to three categories: "The active device," "Not the active device," or "I don't know." We report the BI and 95% confidence interval (CI) based on the week 16 responses, both for index respondents alone and for all household respondents. We adjusted the latter CI for the intrahousehold correlation, ρ=0.60, specified a priori. If the correlation were 0.0, no adjustment would be needed and all participants would be independent observations. If the correlation were 1.0, then each household would be treated as only one observation, since all members of a household would be perfectly correlated in their responses. To supplement these analyses, we also tested, via the 95% CI, the within-assignment null hypotheses that the proportions successfully blinded (i.e., those with "don't know" or incorrect responses) were $\leq$0.5. We did not solicit the blinding status of investigators or contractors. We include the analyses showing only the index respondents to represent a situation in which the correlation coefficient is equal to 0.0.

Finally, to evaluate whether unblinding of participants influenced their reporting of HCGI episodes, we stratified by guess group (active, sham, and don't know) and estimated, within strata, rates and incidence rate ratios (IRR) of HCGI for the sham and active devices. These analyses were performed by using guesses from the end of study (week 16) questionnaire.

Health Outcomes

Participants aged $\geq$12 years were asked to record each day in diaries whether they had symptoms such as nausea, vomiting, diarrhea, abdominal cramps, cough, and fever; index respondents were asked to record these data for children and other household members who might need assistance. The principal health outcome measured in the trial was similar to the "highly credible gastrointestinal illness" reported by Payment et al. A new episode, defined before the analysis was performed, was defined as any of the following four conditions, preceded by at least 6 HCGI-free days: 1) vomiting; 2) watery diarrhea; 3) soft diarrhea and abdominal cramps occurring together on any day; or 4) nausea and abdominal cramps occurring together on any day.

Episodes during the first 6 days of the study were also included, without the restriction of 6 disease-free days before the study. If HCGI information was missing for a particular day, that day was evaluated as HCGI-free for the purpose of identifying subsequent episodes of HCGI. HCGI data were analyzed by Poisson regression adjusted for the intrahousehold correlation introduced by the clustered sampling design. We examined the duration of HCGI episodes, in days, by device. The attributable risk for HCGI from drinking water was calculated as (IRR − 1) / (IRR), where IRR is the incidence rate ratio of the rate of HCGI in the sham group compared with that in the active group.

Water Consumption

Water consumption was self-reported by using data collected in questions inserted into the final health questionnaire. Participants were asked to estimate (in numbers of 8-oz. glasses) their consumption of drinking water at home (separately through the study device and through all other sources at home) and outside the home. Participants were provided with water bottles and encouraged to carry water from the home device for use when outside the home. Mean water consumption was compared by study group via the two-sample t-test.

Results

Recruitment and Enrollment

Flyers describing the trial were distributed to 29,415 homes. Of 573 households screened after contacting us for more information, 439 (77%) were ineligible for the trial. The most common reasons for ineligibility included using bottled water (21%) or a home water filter device (13%); no children in the household (17%); and preexisting problems with the kitchen plumbing (14%). Of the 134 eligible households, 47 (35%) declined to participate. We were able to install a treatment device in 80 (92%) of the 87 consenting households. Eighty households were needed to meet the sample size requirements discussed below.

Three households were excluded from the trial after the device was installed: one operated a day-care center in the home; at the second, household members objected to the taste of the water after installation; and at the third, household members failed to submit any health diaries. The remaining 77 households (38 active; 39 sham) with 236 participants (118 active; 118 sham) provided partial or complete data on blinding and health outcomes and form the basis for the analyses presented in this report.

Completeness of Health Data Collection from Participants

For each participant, the maximum number of health diaries that could be collected was eight (biweekly over 16 weeks) with 112 possible days of data (16 weeks times 7 days). Seventy-four (96%) of the 77 households completed all 16 weeks of the trial. In the active group, 879 (85%) biweekly questionnaires were received from a possible 1,032 questionnaires. In

the sham group, 861 (89%) of a possible 968 questionnaires were received. In the diaries received, health data were provided for 91% of possible days by participants in the active group and for 86% in the sham group.

Randomization and Baseline Characteristics (Table 1)

The groups were comparable at baseline as measured by the distribution of age, gender, health status, and preexisting gastrointestinal complaints. The average number of participants per household in the sham group was 3.03 and in the active group was 3.11 (p=0.80). The average number of children <12 years of age in each household was 0.73 in the sham group and 0.75 in the active group (p=0.86). Of the index respondents, 67% were female.

Water Consumption (Exposure) Patterns during the Trial

Participants in the sham group reported drinking an average of 3.1 glasses of unheated water per day from the study device, and those in the active group drank 3.0 glasses per day (p=0.73). There was no difference in the total amount of drinking water consumed by the participants from all sources (mean 6.8 glasses per day in the sham group; 7.4 glasses per day in the active group, p=0.46).

Effectiveness of Blinding of Participants (Table 2)

The blinding index was 0.64 (95% CI 0.51-0.78) when the week 16 questionnaires of 145 participants ≥12 years of age were analyzed (Table 2). This finding, adjusted for an intra-household correlation (ρ) of 0.60 was highly robust to the choice of correlation coefficients: at ρ=0.40 the CI widens by 0.02, and at ρ=0.80 it narrows by 0.02. The blinding index was 0.65 (95% CI 0.53-0.76) when the 64 index participants were analyzed. Overall, most subjects guessed "active" as their device assignment (50%); 33% guessed "don't know," and the rest guessed "sham."

Within device group, 83% (95% CI 74%-92%) of participants assigned to the sham group appeared to be successfully blinded (i.e., guessed "don't know" or "active"), compared with 43% (95% CI 32%-54%) of those assigned to the active group. Results among index participants were similar to the overall findings.

Analysis of Gastrointestinal Illnesses (Tables 3 and 4)

In the sham group there were 103 episodes of HCGI and 10,790 days on which these subjects were at risk for HCGI (3.48 episodes per person-year; adjusted 95% CI 2.26,-5.34). In the active group there were 82 episodes of HCGI during 11,380 days at risk (2.63 episodes per person-year; adjusted 95% CI 1.82, 3.79). The IRR was 1.32 (adjusted 95% CI 0.75, -2.33) when all household respondents were analyzed and 1.09 (95% CI 0.63, -1.90) when data were analyzed only from the index respondent in each household. Data were also analyzed for the component definitions based on the first day of each episode of HCGI (vomiting, watery diarrhea, soft diarrhea with abdominal cramps, and nausea with abdominal cramps) (Table

Table 1. Baseline characteristics of 236 participants in Pilot Water Evaluation Trial

Characteristic	Sham (n = 118)	Active (n = 118)
Age (Years)	n (%)	n (%)
≤11	28 (24)	29 (25)
12-19	10 (8)	13 (11)
20-29	9 (8)	4 (3)
30-39	22 (19)	18 (15)
40-49	21 (18)	24 (20)
50-59	16 (14)	14 (12)
≥60	12 (10)	16 (14)
Sex (%)	n (%)	n (%)
Female	57 (48)	56 (48)
Male	61 (52)	62 (52)
Prior medical conditions	n (%)	n (%)
Crohn's disease	1 (1)	0 (0)
Diverticulitis	1 (1)	3 (3)
Frequent heartburn	5 (4)	8 (7)
Irritable bowel syndrome	7 (6)	2 (2)
Milk intolerance	4 (3)	5 (4)
Stomach ulcer	5 (4)	4 (3)
Ulcerative colitis	0 (0)	1 (1)
Migraine headaches	14 (12)	13 (11)
Self-assessment of current health	n (%)	n (%)
Excellent	42 (36)	41 (35)
Very good	54 (46)	53 (45)
Good	20 (17)	20 (17)
Fair	2 (2)	4 (3)
Poor	0 (0)	0 (0)
Current medical conditions (prior 7 days)	n (%)	n (%)
Abdominal cramps	19 (16)	15 (13)
Diarrhea	14 (12)	13 (11)
Nausea	16 (14)	11 (9)
Vomiting	2 (2)	3 (3)
Fever	6 (5)	5 (4)
Pregnant	1 (1)	1 (1)

3). If drinking water were the cause of the reported increase in gastrointestinal disease, the adjusted rate ratio for episodes of HCGI would suggest an attributable risk of 0.24 (95% CI -0.33, -0.57).

HCGI episodes were typically brief; they did not differ in duration between the two groups (p=0.23). The median duration of episodes in the active group was 1 day (range 1 to 40 days; interquartile range 1 to 2 days). The median duration of episodes in the sham group was 2 days (range 1 to 40 days; interquartile range 1 to 3 days).

Among those guessing that they were using a sham device and also among the group of participants guessing "don't

Table 2. Final (Week 16) Device Blinding Questionnaire, Pilot Water Evaluation Trial

All participants (≥12 years of age) who completed final questionnaire

Guess	Sham device group (%)	Active device group (%)	Total (%)
Sham	12 (17.4)	12 (15.8)	24 (16.6)
Active	30 (43.5)	43 (56.6)	73 (50.3)
Don't know	27 (39.1)	21 (27.6)	48 (33.1)
Total*	69 (100.0)	76 (100.0)	145 (100.0)

Index respondents only+

Guess	Sham device group (%)	Active device group (%)	Total (%)
Sham	5 (16.1)	5 (15.2)	10 (15.6)
Active	13 (41.9)	19 (57.6)	32 (50.0)
Don't know	13 (41.9)	9 (27.3)	22 (34.4)
Total	31 (100.0)	33 (100.0)	64 (100.0)

*Does not include 21 participants from the sham device group and 13 participants from the active device group who did not complete the final blinding questionnaire.

+Blinding index for all participants (adjusted for intrahousehold correlation, ρ=0.60)=0.64 (95% confidence interval [CI] 0.51–0.78). Blinding index for index respondents alone = 0.65 (95% CI 0.53–0.76).

know" the reported rates of HCGI were nearly identical in the two device groups (Table 4). However, among subjects guessing that they were using an active device, the rates of illness were higher among those actually using the sham. A similar pattern (higher rate in the sham group) was seen among subjects who did not complete a final blinding questionnaire.

Quality Control

Early in the trial we learned that five devices (two active and three sham) had been installed in reverse. The normal flow of water in the device is through the filter first and then through the UV light chamber. In these five devices, the flow passed through the UV chamber first and then through the filter. For all potentially reversed devices (i.e., those installed before the discovery of this reversal), we either directly inspected them or inspected photographs obtained at installation as part of our routine quality control procedures. Although these devices still provided treatment of water, they had not been installed according to protocol and were replaced with identical devices (sham or active) connected correctly. We have retained these households in our analyses.

Discussion

This pilot study is the first in the United States to evaluate blinding in a randomized, controlled trial of drinking water. Our findings suggest that at least two thirds of participants remained blinded to device assignment throughout the 16-week trial. The actual level of blinding was probably greater, since some subjects may have guessed their device assignment by chance alone.

Our trial was undertaken as the first step in planning a larger trial to evaluate the risk for infection from drinking tap water fully treated to meet conventional regulatory standards in the United States. Without the ability to blind the participants in such intervention trials, the results of any subsequent larger studies intended to evaluate health effects attributable to drinking water would remain controversial. Our data suggest that subjects were effectively blinded throughout the pilot trial. We estimated that a higher proportion of subjects was blinded in the sham group (83%) than in the active group (43%); however, in the active group the 95% CI included 50%, indicating that correct responses may be attributable to chance.

A secondary goal of the trial was to compare gastrointestinal illness rates in the two groups. Although the rate of gastrointestinal illness was higher in the sham group than in the treatment group, this difference was not statistically significant. The relative rates of illness observed overall and in specific subgroups (gender and age) were very similar to those reported in an earlier, larger randomized trial in Canada, which found a statistically significant difference between the active and control groups (1). Preliminary results from a similar trial in Australia, which also was blinded, found no difference in the rates of disease in the active and sham groups (7).

Effectiveness of Participant Blinding

Despite the widespread use of participant blinding in intervention trials, little methodologic literature is available with

Table 3. Episodes[a] of highly credible gastrointestinal illness (HCGI) and days of illness, Pilot Water Evaluation Trial

	Sham device group	Active device group	Total
Total episodes of HCGI, defined by[b]	103	82	185
Vomiting	18	30	48
Watery diarrhea	73	42	115
Soft diarrhea with abdominal cramps	7	6	13
Nausea with abdominal cramps	16	17	33
Total days of HCGI, defined by[b]	261	190	451
Vomiting	35	78	113
Watery diarrhea	207	99	306
Soft diarrhea with abdominal cramps	8	8	16
Nausea with abdominal cramps	31	30	61
Total days at risk for HCGI episodes	10,790	11,380	22,170
Total days of observation	11,642	12,036	23,678

[a]A new episode was defined as the presence of any of four definitions of HCGI, preceded by 6 HCGI-free days. The difference in total episodes of HCGI was the principal a priori health outcome measure for the study.

[b]Because individual participants could report multiple definitions of HCGI on the same day, the total episodes of HCGI (and total days of HCGI) are less than the sums of the individual definitions.

Table 4. Rates of highly credible gastrointestinal illness (HCGI) episodes and incidence rate ratios (IRR)[a] for all respondents and index respondents, by respondent guess about device assignment, week 16,[b] Pilot Water Evaluation Trial

	All Repondents		Index Respondents	
	Sham device	Active device	Sham device	Active device
Guess = "Sham"				
Episodes of HCGI	4	4	3	1
Person-time (person-years)	3	3	1	1
No. of respondents	12	12	5	5
Rate (95% CI)	1.2 (0.3–4.8)	1.1 (0.5–2.7)	2.1 (0.7–6.6)	0.7 (0.1–4.8)
IRR for sham vs. active (95% CI)	1.0 (0.2–5.4)		3.16 (0.3–30.4)	
Guess = "Active"				
Episodes of HCGI	30	30	6	15
Person-time (person-years)	8	12	4	5
No. of respondents	30	43	13	19
Rate (95% CI)	3.6 (2.1–6.3)	2.5 (1.3–4.5)	1.6 (0.7–3.6)	2.8 (1.7–4.6)
IRR for sham vs. active (95% CI)	1.5 (0.7–3.3)		0.6 (0.2–1.5)	
Guess = "Don't know"				
Episodes of HCGI	18	15	10	6
Person-time (person-years)	8	6	4	3
No. of respondents	27	21	13	9
Rate (95% CI)	2.4 (1.2–4.6)	2.5 (1.1–5.5)	2.7 (1.5–5.1)	2.3 (1.0–5.2)
IRR for sham vs.active (95% CI)	1.0 (0.3–2.7)		1.2 (0.4–3.3)	
All guesses				
Episodes of HCGI	52	49	19	22
Person-time (person-years)	19	22	9	9
No. of respondents	69	76	31	33
Rate (95% CI)	2.7 (1.7–4.3)	2.2 (1.5–3.4)	2.2 (1.4–3.4)	2.3 (1.5–3.5)
IRR for sham vs.active (95% CI)	1.2 (0.6–2.2)		0.9 (0.5–1.7)	
No guess given				
Episodes of HCGI	12	5	5	2
Person-time (person-years)	4	2	1	1
No. of respondents	21	13	8	5
Rate (95% CI)	3.3 (1.1–9.7)	2.7 (1.1–6.9)	5.4 (2.6–11.3)	3.0 (0.7–11.8)
IRR for sham vs. active (95% CI)	1.2 (0.3–5.1)		1.8 (0.4–8.8)	

[a]Rates of HCGI and IRR were calculated by Poisson regression and were adjusted for the intrahousehold correlation introduced by the sampling design.

[b]Respondents for the blinding questionnaires were all aged ≥12 years.

which to measure its effectiveness. In the absence of successful blinding, biases may explain the results of a trial. For example, subjects aware that they are not receiving an intervention (i.e., the sham group) could, intentionally or not, report a higher (or lower) frequency of disease.

Our measurement of blinding is based on work by James (4). If 100% of the participants in a study guess their treatment assignment correctly, the BI would be 0.0 (complete unblinding). If 50% of the participants guess correctly, the BI would be 0.5 (random guessing). If 100% of the participants were to guess "don't know," the BI would be 1.0 (perfect blinding). Our trial provides evidence that blinding, as measured by the blinding index, can be maintained successfully during an in-home

drinking water intervention trial. Subjects in both groups were more likely to guess that they had the active device; we speculate that this may be related to the fact that both active and placebo devices warmed the water during long periods when water was not being drawn from the tap. This warming could have led participants in both groups to believe they were using the active device. Our study lasted only 4 months, and the effectiveness of blinding may decline during a prolonged trial. Drinking water intervention trials conducted for extended periods should also assess blinding throughout the trial. The frequency of such questioning of participants should be designed to avoid raising awareness of treatment group assignment, which might increase unblinding.

Comparison with Payment's Prior Intervention Trial

The rates of illness we observed (as measured by HCGI) were higher than those reported in the earlier work of Payment et al.(1). Our trial was much shorter than Payment's (4 months vs. 15 months). Conceivably, subjects in both active and sham groups are more likely to report (or even overreport) illness early in the trial, when enrollment and participation instructions have been recently given and emphasized. Another possible explanation for the difference we observed in the rates of illness between the groups could be that certain persons contributed a disproportionate number of illnesses. However, our data did not support this explanation, since the distribution of number of episodes did not differ between the two groups.

We detected no significant differences in water consumption patterns of the two groups. If any differences in consumption of water outside the home did exist, a conservative bias would have been introduced into our results that would likely have attenuated any difference in observed health effects.

Although our definition of HCGI was patterned after the work of Payment et al. (1), there were some differences. In the earlier work, symptoms were reported to the index respondent, who completed all the questionnaires for all subjects. In our study, each participant aged ≥12 years completed his or her own health questionnaires. Payment et al. excluded episodes believed to have other plausible etiologies; we included all episodes. We asked participants to indicate diarrhea on days in which they had two or more loose stools; Payment et al. do not specify the definition of diarrhea. Finally, Payment et al. used the term "liquid" stool; our term was "watery."

Payment's point estimate of the effect (rate ratio = 1.38) is similar to ours (rate ratio = 1.32). Payment reported an attributable fraction of 35% (of HCGI attributable to drinking water consumption); our study's point estimate of the attributable fraction was 24%.

Limitations

We selected for the trial only families who owned their homes so that consent would be needed only from the participating family and not also from a landlord. This selection may have led to the recruitment of subjects of higher socioeconomic status than the target population. However, any bias would not affect the internal validity of the study because the subjects were randomized.

Knowledge of experimental group assignment can influence self-reported endpoints in clinical trials, thereby reducing the internal validity of the findings. The experimental group assignment might be revealed to participants through distinguishing features of the intervention (e.g., after installation of the filter, the household water tastes different), through accidental communication of the assignment by study personnel (e.g., the plumber), and, especially in trials with long follow-up, through early or repeated occurrence of an episodic outcome or its symptoms (e.g., HCGI).

Several limitations should be considered in interpreting the health results of this trial. First, it was conducted in a single municipality that received its water from a challenged surface water source and treated water with chloramination. As is typical of randomized, controlled trials, our study relied on volunteers, which hampers external generalizations. As a result of randomization, however, its strength lies in its internal validity (enabling comparison of active and sham groups without fear of selection bias). Data from a series of studies of various designs conducted in various locations are necessary for the development of a national estimate of waterborne disease. This is the approach being used by CDC and EPA. Finally, we provided a treatment device for only one tap in each household. If participants obtained drinking water from other taps (despite our instructions to avoid this as much as possible), our study may underestimate any attributable risk. Use of devices that treated all water entering each household was neither practically nor economically feasible.

Our sample size in this pilot study was determined based on the blinding index. Our study was not designed to be large enough to detect a difference in health (as measured by HCGI) between the sham and active groups of the magnitude previously reported by Payment. If a study were designed with 80% power to detect a true reduction in HCGI to 1.3 episodes/person-year from a level of 2.6 in the sham group, observation of 200 households (of approximatly three persons per household) would be required for one year of observation (based on a two-sided 0.05-level test adjusted for intrahousehold correlation [$\rho=0.60$]). Additionally, although our study did not collect the data necessary to evaluate the severity of the HCGI episodes, our data indicate that about half the illnesses in both groups were short-lived (only 1 or 2 days long). We suggest that future studies include measurement of episodes associated with lost time at work or school or resulting in calls or visits to physicians, clinics, or emergency rooms. Such measurement will allow better assessment of the public health impact of any differences attributable to drinking water consumption.

One theoretical explanation for the results we observed could be that the sham device somehow degraded the drinking water. In a limited water sampling program (data not shown), we did not find evidence to support this. Additionally, in a large study with the same device in Australia, no difference in health effects was found between the active and sham device groups, suggesting that degradation of the water by the sham device is not a likely explanation for our findings (7).

Finally, drinking water proceeds in a complicated path from environmental sources, through water treatment and distribution systems, through internal pipes in the home, and eventually to a consumer's tap. Drinking water intervention trials that use in-home treatment devices cannot isolate the source of any specific site of contamination. Rather, such trials can only help provide evidence to suggest whether further evaluation of the drinking water pathway may be necessary in specific settings.

Conclusion

Our data suggest that subjects were effectively blinded throughout a 4-month trial of an in-home drinking water intervention. Although the rate of gastrointestinal illness was higher in the sham group than in the treatment group, this difference was not statistically significant, and the trial was not designed to detect a difference of the magnitude observed. The relative rates of illness overall were very similar to those reported in an earlier, larger randomized trial in Canada, which did report statistically significant differences in HCGI between the groups. Our findings suggest that it will be possible to conduct larger blinded, randomized trials to evaluate health effects related to tap water consumption.

Acknowledgments

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Address for correspondence: John M. Colford, Jr.; UC Berkeley School of Public Health, 140 Warren Hall #7360, Berkeley, CA 94720, USA; fax: 413-228-5931; jcolford@socrates.berkeley.edu

A Large Outbreak of Legionnaires' Disease at a Flower Show, the Netherlands, 1999

Jeroen W. Den Boer,*† Ed P.F. Yzerman,‡ Joop Schellekens,* Kamilla D. Lettinga,§ Hendriek C. Boshuizen,* Jim E. Van Steenbergen,¶ Arnold Bosman,* Susan Van den Hof,* Hans A. Van Vliet,* Marcel F. Peeters,# Ruud J. Van Ketel,§ Peter Speelman,§ Jacob L. Kool,* and Marina A.E. Conyn-Van Spaendonck*

In 1999, an outbreak of Legionnaires' disease affected many visitors to a flower show in the Netherlands. To identify the source of the outbreak, we performed an environmental investigation, as well as a case-control study among visitors and a serologic cohort study among exhibitors to measure exposure to possible sources. Of 77,061 visitors, 188 became ill (133 confirmed and 55 probable cases), for an attack rate of 0.23% for visitors and 0.61% for exhibitors. Two whirlpool spas in halls 3 and 4 of the exhibition and a sprinkler in hall 8 were culture positive for *Legionella pneumophila*. One of three genotypes found in both whirlpool spas was identical to the isolates from 28 of 29 culture-positive patients. Persons who paused at the whirlpool spa in hall 3 were at increased risk for becoming ill. This study illustrates that whirlpool spas may be an important health hazard if disinfection fails.

From March 7 to 11, 1999, 10 patients with severe pneumonia were admitted to a hospital in Hoorn, in the northern part of the Netherlands. The clinical condition of these patients deteriorated quickly and unexpectedly, with eight requiring mechanical ventilation. On March 11, six of the eight patients were diagnosed with Legionnaires' disease on the basis of a positive *Legionella* urine antigen test. Additional patients with severe pneumonia were sent to another hospital since all the respirators at the Hoorn hospital were in use. Two of these patients were subsequently diagnosed with Legionnaires' disease by urine antigen test.

An exploratory case-control study using a questionnaire on exposure to potential sources was conducted; six confirmed and four probable cases were included. All patients and 3 of 21 controls had visited the West Frisian Flower Show (p<0.001). Since no other environmental risk factor was identified, the outbreak investigation focused on the flower show. This show is an annual event that includes an agricultural and consumer products exhibition, located in the nearby town of Bovenkarspel. Held from February 19 to 28, the flower show attracted 77,061 visitors. After a nationwide warning was issued to ensure detection and appropriate treatment of any additional cases, it became clear that >180 persons had been

affected. We report how the source of the outbreak was identified by a combination of an environmental investigation, a case-control study among flower show visitors, and a cohort study among >700 exhibitors.

Methods

A confirmed case of Legionnaires' disease was defined as radiologically confirmed pneumonia in a visitor to the exhibition or a member of the exhibition staff, with onset no earlier than February 19, 1999, and no later than March 21, 1999, as well as laboratory evidence of *Legionella pneumophila* infection. Laboratory evidence included isolation of *L. pneumophila* from respiratory secretions, detection of *L. pneumophila* antigens in urine, or a fourfold or higher rise in antibody titers to *L. pneumophila* in paired acute- and convalescent-phase sera, as reported by clinicians. A probable case was defined as radiologically confirmed pneumonia with onset no earlier than February 19, 1999, and no later than March 21, 1999, in an exhibition visitor or a member of the exhibition staff who did not meet laboratory criteria for a confirmed case, but who had no laboratory evidence of infection by other microorganisms. All local health services and hospitals in the Netherlands were informed of these criteria and asked to report cases of pneumonia in persons who had visited the exhibition. Unsolicited case reports from the public were also recorded. Finally, all Dutch clinical medical microbiology laboratories were asked to send clinical *Legionella* isolates from flower show-related Legionnaires' disease patients to the National Institute of Public Health and the Environment (RIVM) for serotyping and genotyping.

*National Institute of Public Health and the Environment, Bilthoven, the Netherlands; †Municipal Health Service Zuid-Kennemerland, Haarlem, the Netherlands; ‡Regional Laboratory of Public Health Haarlem, Haarlem, the Netherlands; §Academic Medical Center, Amsterdam, the Netherlands; ¶National Outbreak Structure for Infectious Diseases, Den Haag, the Netherlands; and #Regional Laboratory of Public Health Tilburg, Tilburg, the Netherlands

Environmental Risk Assessment

A map of the water system at the exhibition site was made to facilitate visual inspection for circulatory dead ends and other potential locations of stagnant water. In addition, we interviewed all exhibitors to compile an inventory of all products using water that had been displayed at the exhibition. Based on these interviews, an 8-point risk-assessment scale was developed to discriminate among the products used during the exhibition. For each of the following items, 1 point was given: use of water; use of water at 20°C to 43°C (the temperature range within which *Legionella* can amplify to dangerous concentrations) (1,2); use of water at 37°C (the optimal temperature for *Legionella* growth) (3); no disinfection of water at 20° to 43°C; no changing of water at 20° to 43°C; occasional misting of water at <60°C; continuous misting of water at <60°C; and substantial surface for misting of water at <60°C.

Two weeks after the end of the exhibition, we began to obtain water and swab samples from all potential sources of *Legionella*. The water samples were concentrated by membrane filtration (0.2 µm), and filtered residues were resuspended in 1 mL sterile water. Of this suspension, 100-µL samples were cultured without dilution and after 10- and 100-fold dilutions on buffered charcoal yeast extract agar with alpha-ketoglutarate (BCYE-alpha) and a selective supplement with dyes and the antibiotics polymyxin, anisomycin, and vancomycin (Legionella MWY Selective Supplement SR118, Oxoid Ltd., Hampshire, England). Plates were incubated at 37°C with increased humidity. In case of bacterial overgrowth, cultures were repeated after pretreatment by heating 30 minutes at 50°C. Swab samples were dispersed by immersion in 1 mL sterile water and cultured as described. Cultures were examined microscopically daily for 14 days. In case of persistent overgrowth, ceftazidime was added to the media. Colonies suspected of being *Legionella* were subcultured to blood agar and BCYE-alpha agar. Identification was confirmed by biochemical tests. *Legionella* isolates were serogrouped by using commercial kits containing antisera against *L. pneumophila* serogroups 1-14, *L. longbeachae* 1 and 2, *L. bozemanii* 1 and 2, *L. dumoffi, L. gormanii, L. jordanis, L. micdadei*, and *L. anisa* (*Legionella* Latex Test, Oxoid Limited, Hampshire, England; *Legionella* antisera "Seiken," Denka Seiken Co. Ltd., Tokyo, Japan). Isolates were genotyped by pulsed-field gel electrophoresis (4) and amplified fragment-length polymorphism (5).

Case-Control Study

To measure visitor exposure to possible sources of *Legionella*, we used a questionnaire, a set of situational drawings, and a floor plan of the exhibition site. The questionnaire addressed health status and details about visits to different parts of the exhibition or displays of devices capable of spreading *Legionella* (6-8). As controls, a random sample of 2,500 men and 2,500 women born before 1960 were selected from the municipal population register. A request for participation in the study was sent to all these persons, but they were asked to reply only if they had visited the exhibition. Of the first 469

who replied, all 196 men and a random selection of 203 women were sent the questionnaire. Respondents were excluded as controls if they had symptoms of respiratory infection within 20 days of their exhibition visit (for pneumonia and bronchitis) or within 4 days of their visit (for influenzalike illness). Both cases and controls were asked to indicate the route they had walked and the stands they had visited on a floor plan of the exhibition site and situational drawings of stands at which devices using water were displayed. To avoid bias, drawings of stands at which products were demonstrated that did not use water were also included. Ill persons were interviewed personally or by proxy.

Variables that were significant in univariate analysis were entered in a multiple logistic regression model. With the use of backward elimination, independent predictors of becoming ill, adjusted for age and sex, were established. Variables were retained in the model if the likelihood ratio test was significant (p<0.1).

Cohort Study

Letters were sent to exhibition volunteers, staff of the company organizing the exhibition, and all exhibitors (n=1,616) (with the exception of persons with confirmed and probable cases), asking them to complete a questionnaire regarding their health status before and after the exhibition. Questions were included about principal work location during the exhibition. Participants were asked to have paired blood samples drawn and sent to RIVM. The first samples were taken by the end of March and the second by mid-May. Serum immunoglobulin (Ig) M and IgG antibodies against *L. pneumophila* were detected by indirect enzyme-linked immunosorbent assay (Virion-Serion, Würzburg, Germany). For every 63-cm^2 area of the exhibition site, the geometric mean IgM and IgG titers of the nearest 35 respondents were determined and plotted after smoothing by using the highest titer in paired sera for each respondent. The correlation of the logs of the antibody titers and distance to aerosol-generating devices was analyzed by linear regression, after the data were adjusted for age and sex of respondents, smoking status, underlying disease, and time worked in each hall. In all analyses, data were included only from persons who had worked at the exhibition after February 22.

Results

A total of 188 cases of Legionnaires' disease (133 confirmed, 55 probable) were reported in visitors (178) and exhibitors (10), originating from throughout the Netherlands. An additional 21 patients had physician-diagnosed pneumonia but no radiologic studies, and 9 patients had positive urine antigen tests but insufficient clinical data. Dates of onset ranged from February 25 to March 16 (Figure 1). The median age of patients was 66 years (range 20 to 91 years), and the male:female ratio was 1:1.4. Diagnosis was confirmed by culture in 29 cases, urine antigen test in 100 cases, and serologic testing in 53 cases (Table 1). In 54 cases, two or more tests were positive. Among patients for whom data were available
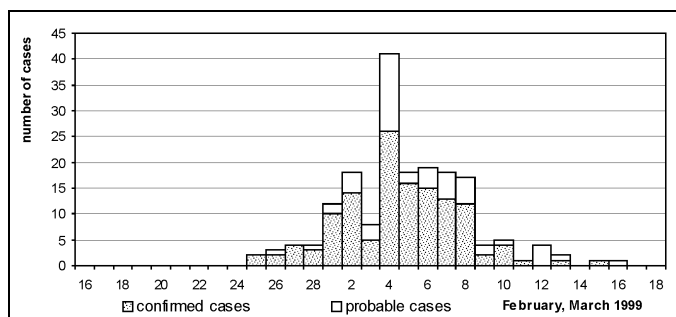
Figure 1. Dates of onset of illness in 186 cases of Legionnaires' disease, February 16–March 18, 1999.
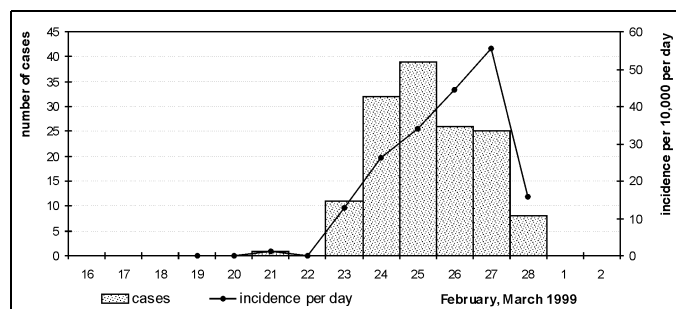
Figure 2. Confirmed and probable cases of Legionnaires' disease per day of visit to flower show. Incidence per 10,000 visitors per day of visit, February 16–March 2, 1999.

and who visited the exhibition once (n=136), the reported incubation period was 2 to 19 days (median 7 days); in 22 cases (16%) the time before onset of illness exceeded 10 days. All but one ill person had visited the exhibition after February 22. The exception was a 55-year-old woman with a history of chronic obstructive pulmonary disease and pneumonia who visited the exhibition on February 21. She visited hall 3 for 45 minutes and stopped near the whirlpool spa.

The attack rate for visitors was 178/77,061 or 0.23%. The daily attack rate increased from 0.011% on February 21 to 0.56% on February 27 (Figure 2). Ten of the exhibitors, volunteers, and employees became ill, for an attack rate of 0.61% among staff. Seven of these 10 staff members worked in the right side of hall 3 (Figures 3a and 3b). The attack rates for staff were 2.7% for hall 3 and 0.4% for hall 4 (Fisher exact test, p=0.005).

Of the 188 patients, 163 (87%) were hospitalized and 34 (21%) required mechanical ventilation. Seventeen persons with confirmed and 4 with probable LD died, for a case-fatality rate of 11%. The case-fatality rate was highest (17%) in patients >70 years of age.

Clinical isolates of 29 patients were available for genotyping. Twenty-eight were identical to the strain designated B-1, and one was identical to B-2.

Environmental Risk Assessment

The 11 halls of the exhibition site were supplied with water by two separate systems. The flower show was held in halls 2,

5, and 13; the consumer exhibition in halls 3 and 4; and the agricultural exhibition in halls 8 and 9 (Figure 4a). During the 3 months before the exhibition, the right side of hall 3 had been partitioned off to preserve flower bulbs at 30°C. In halls 5 and 13, 11 decorative fountains and a waterfall were installed. The temperature in these two halls was kept under 15°C to preserve the flowers. Our risk assessment of the water-using devices showed (Figure 4b) that a whirlpool spa in hall 3 posed the greatest risk (8 points), followed by a whirlpool spa in hall 4 (6 points), two bubblemats[1] in halls 3 and 4 (4 points), 11 fountains in halls 5 and 13 (4 points), and a sprinkler installation in hall 8 (3 points). None of these devices were maintained with adequate disinfection. The whirlpool in hall 3 had never been used before, and its disinfection system failed. Ten samples were collected from the municipal water supply and 127 from the water system of the exhibition building (Figure 4a). Of 27 water-using devices that had been on display at the exhibition, 12 (including the whirlpool spas and the sprinkler installation) were available and still contained water (Figure 4c). A total of 145 samples were taken from these 12 devices. All cultures of specimens from the water supply system were negative for *L. pneumophila*, but the organism was cultured from paper filters of the whirlpool spa in hall 3 (>100 colonies), the whirlpool spa in hall 4 (2 colonies), and the sprinkler installation in hall 8 (15 colonies). Subsequent sampling of the inner tubing system

[1]A bubblemat is an inflatable rubber mat that causes a whirlpool-like effect when placed in a normal bathtub.

Table 1. Positive diagnostic tests results for 188 cases (133 confirmed and 55 probable) of Legionellosis, the Netherlands, 1999[a]

	Culture	Urinary antigen	Fourfold rise in titer	Direct immunofluorescence	PCR[b]	Single high titer
Culture	29	24	3	2	11	4
Urinary antigen	24	100	25	1	15	12
Fourfold rise in titer	3	25	53	0	1	0
Direct immunofluorescence	2	1	0	2	1	0
PCR	11	15	1	1	18	3
Single high titer	4	12	0	0	3	18

[a]The table is read as follows: of 29 patients who had positive cultures, 24 were positive by urinary antigen, 3 had a fourfold rise in titer, 2 were positive by direct immunofluorescence, and 11 by PCR, and 4 had single high titers.

[b]PCR = polymerase chain reaction.
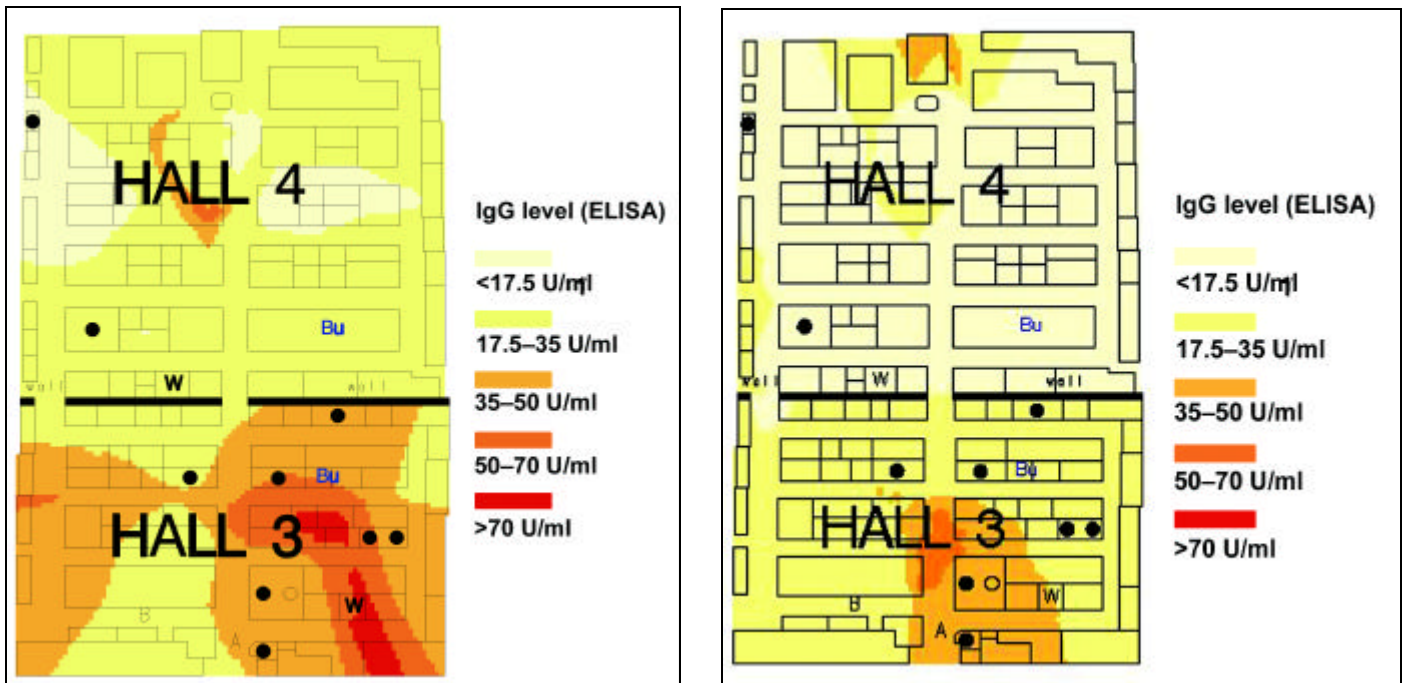
Figure 3a. Smoothed mean geometric immunoglobulin (Ig) M antibody titers to *Legionella pneumophila* of nearest 35 exhibitors in hall 3 and 4 per 63 cm² of exhibition area; confirmed and probable cases among exhibitors in halls 3 and 4. l = confirmed case in exhibitor; m = probable case in exhibitor; Bu = bubblemat; W = whirlpool spa. Figure 3b. Smoothed mean geometric IgG antibody titers to *L. pneumophila* of 35 exhibitors nearest to whirlpool in halls 3 and 4 per 63 cm² of exhibition area; exhibitors ill with confirmed and probable cases in halls 3 and 4.● = confirmed exhibitor case; ○ = probable exhibitor case; Bu = bubblemat; W = whirlpool spa.

of both whirlpool spas, 6 weeks after the exhibition ended, yielded abundant growth of *L. pneumophila* from swabs of the hall 3 whirlpool spa. No growth was found in the remaining water from the whirlpool spa in hall 4, which had a chlorine concentration of 0.64 mg/L.

Serotyping and genotyping of environmental isolates yielded three distinct genotypes: two serogroup 1 isolates (called B-1 and B-2) and one serogroup 6 isolate (called B-3). Genotyping results for pulsed-field gel electrophoresis and amplified fragment-length polymorphism were in agreement (Figure 5). The filter and the inner tubing of the hall 3 whirlpool spa contained B-1, B-2, and B-3 (semiquantitatively B1>B2>B3), the filter of the hall 4 whirlpool spa contained B-1, and the filter of the sprinkling installation contained B-2 and B-3 (semiquantitatively B-2>B-3). Cultures of the inner tubing of the hall 3 whirlpool spa taken several months after the exhibition were still positive and yielded all three genotypes.

Case-Control Study

The rates of response to the questionnaire and drawings were 85% and 58% for cases and 98% and 65% for controls, respectively. Thirty-six controls who reported symptoms of respiratory infection were excluded from the analysis. Analysis was restricted to data from 71 confirmed and 30 probable cases and 119 controls who visited the exhibition after February 22 (Table 2). The variables that remained in the multiple regression model were based on 62 confirmed and 21 probable cases and 105 controls (Table 3). Our analysis showed that smoking and length of stay at the exhibition were risk factors for infec-

Table 2. Results of univariate analysis of data from questionnaires and drawings comparing host factors and visits with specific sites at the exhibition for cases and controls.

Study population	Respondents' questionnaire and set of drawings; univariate OR[a] (95% CI) (101 cases, 119 controls)	Respondents' questionnaire and set of drawings; raw data (101 cases, 119 controls)
Age	1.1 (1.0 – 1.1)	na
Male	2.7 (1.6 – 4.7)	63/45
Underlying disease	7.2 (1.5 – 33.1)	11/2
Smoking	2.0 (1.1 – 3.6)	42/31
Total hours at exhibition	1.7 (1.4 – 2.1)	4/3
Hours at consumer exhibition	1.0 (0.8 – 1.3)	1/1
Pausing at whirlpool spa in hall 3	4.2 (1.9 – 9.0)	41/21
Pausing at bubblemat in hall 3	3.7 (1.6 – 8.2)	37/17
Pausing in gangway of bubblemat in hall 3	0.4 (0.2 – 1.0)	24/35
Pausing at electric kettle in hall 3	3.0 (1.3 – 7.0)	26/12
Pausing at whirlpool in hall 4	2.4 (1.1 – 5.4)	31/20
Pausing at steam iron in hall 4	5.4 (1.4 – 22.0)	16/3

[a]OR = odds ratio; CI = 95% confidence intervals; na = not available.

Table 3. Logistic regression model of data from questionnaires and drawings comparing host factors and visits to specific sites at the exhibition for cases and controls

Study population	Respondents to questionnaire and set of drawings; OR[a] (95% CI)(101 cases, 119 controls)
Age	1.1 (1.0 – 1.1)
Male sex	2.1 (1.0 – 4.5)
Smoking	6.0 (2.4 – 15.1)
Total hours at exhibition	2.2 (1.5 – 3.2)
Hours at consumer exhibition	0.5 (0.3 – 0.8)
Pausing at whirlpool spa in hall 3[b]	2.6 (1.1 – 6.6)
Pausing at bubblemat in hall 3[b]	3.0 (1.1 – 8.0)
Pausing in gangway of bubblemat in hall 3[b]	0.3 (0.1 – 0.8)

[a]OR = odds ratio; CI = 95% confidence interval.
[b]See Figure 3.

tion. Length of stay exclusively at the consumer products exhibition showed an inverse relation, after data were adjusted for total length of stay. However, cases and controls on average spent the same amount of time at the consumer exhibition. Drinking tap water was not a risk factor. Visiting the whirlpool spa display in hall 3 was a risk factor. Visiting the bubblemat display in hall 3 was also associated with risk, but visiting a display of the same bubblemat in the gangway showed an inverse relation. Similar but not always statistically significant results were found when the analysis was limited to confirmed cases.

Table 4. Characteristics of persons with legionellosis who worked in the right half of hall 3 compared with staff members who did not become ill and who worked in the same hall

Age group	Cases in right half of hall 3 (n=7) n (%)	Cohort respondents in hall 3 (n=151) n (%)
<30	0 (0)	23 (15.7)
30-39	0 (0)	24 (16.3)
40-49	1 (14)	29 (19.7)
50-59	4 (57)	46 (31.3)
60-69	2 (29)	20 (13.6)
≥70	0 (0)	5 (3.4)
Males	6 (86)	67 (45.3)
Smokers	4 (67)[a]	44 (29.1)
Immunocompromised	0 (0)	5 (3.3)
History of pneumonia	1 (14)	5 (3.3)
History of diabetes	1 (14)	5 (3.3)

[a]Data missing for one case.
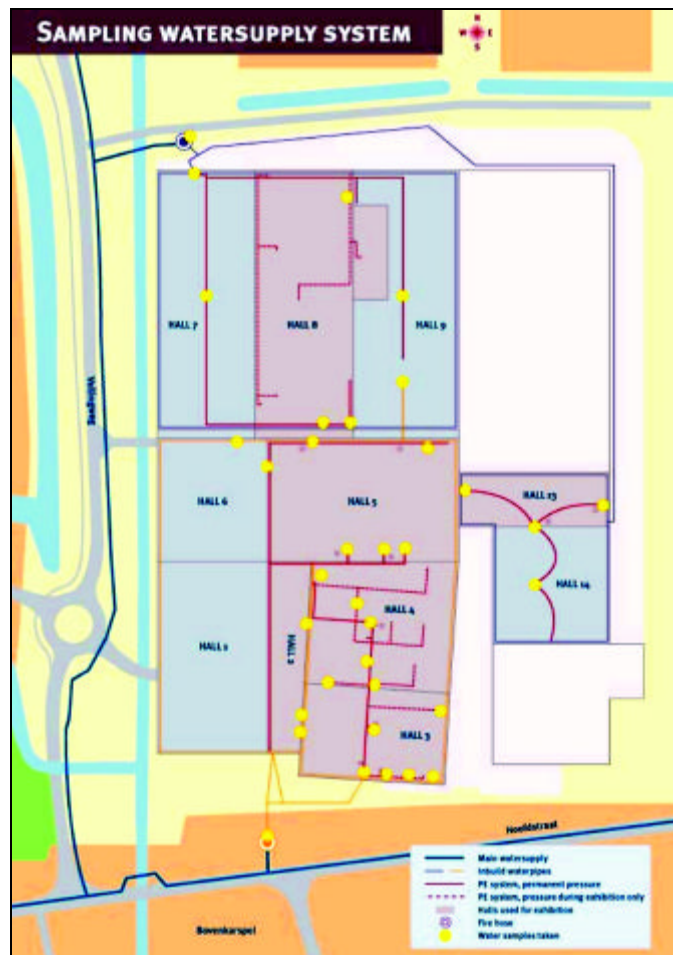
Figure 4. Water-supply system for exhibition hall, Bovenkarspel the Netherlands, 1999. PE = polyethylene.

Cohort Study

Of the exhibition staff, 880 responded to the cohort study questionnaire (54%), and 714 (44%) provided two analyzable blood samples. Geometric mean IgG and IgM titers were not associated with drinking tap water or contact with potting compost. Geometric mean IgM and IgG titers were significantly increased (p<0.0002) among exhibitors in hall 3 but not among those in hall 4, compared with exhibitors working in other halls. Respondents who worked in the right side of hall 3 had the highest average antibody titers (Figures 3a and 3b). Multiple linear regression showed that proximity to both the whirlpool spa and the bubblemat in hall 3 was positively associated with increase in antibody titer, but no such relation was found for distance to the bubblemat and whirlpool spa in hall 4. Since the bubblemat and the whirlpool spa in hall 3 were situated close together, exposure to each was highly correlated and risk could not be differentiated. An inverse relation was found between the attack rate for staff with confirmed legionellosis and the distance of their workplace to the whirlpool in hall 3 (p=0.0009). Staff members who became ill and who worked in the right side of hall 3 differed from their colleagues in the same hall with respect to age, gender, and smoking habit (Table 4).
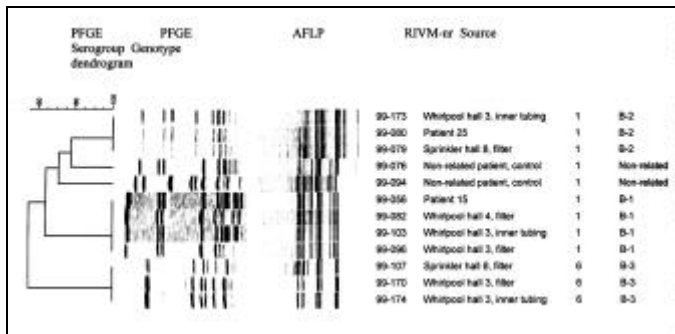
Figure 5. Pulsed-field gel electrophoresis (PFGE) and amplified fragment-length polymorphism (AFLP) patterns of a representative selection of clinical and environmental *Legionella pneumophila* isolates; the dendrogram shows clustering in PFGE. The AFLP and PFGE pattern of the isolate of patient 15 (genotype B-1) was found in 28 of the 29 isolates of culture-positive cases; the same pattern was found in isolates cultured from the whirlpool spas in halls 3 and 4. The AFLP and PFGE pattern of the isolate of patient 25 (genotype B-2) was unique among culture-positive cases; the same pattern was found in isolates cultured from the whirlpool spa in hall 3 and the sprinkler.

Discussion

A new whirlpool spa, within 4 days after its installation, was the major source of one of the world's largest outbreaks of Legionnaires' disease. With 188 (133 laboratory-confirmed) cases, this outbreak is only exceeded by the original 1976 outbreak in Philadelphia (221 cases). Because our case definition for probable cases was broad, the 55 probable cases may include some persons with other, undetected causes of pneumonia. Detailed studies of clinical and laboratory-diagnostic characteristics of the patients are ongoing.

Despite these limitations, this outbreak of *Legionella* pneumonia is certainly the largest to be associated with a contaminated whirlpool spa. Although the overall attack rate (0.24%) was low for this outbreak in comparison with other nonhospital indoor outbreaks (4% to 7%) (9-11), the large number of visitors resulted in a large number of patients. The fact that the outbreak was not detected until 14 days after the first case of pneumonia was diagnosed, when 71 pneumonia patients had already been hospitalized, is remarkable. In hindsight, the first hospitalized patient could have been diagnosed on February 25, when only 40 to 50 of the eventual 188 patients had been infected. Although immediate diagnosis would have enhanced the possibility of timely public health intervention, the source of the outbreak is unlikely to have been discovered before the end of the exhibition. Late detection may be due partly to small-scale use of the *Legionella* urine antigen test in the Netherlands. Dutch physicians may have considered Legionnaires' disease a rare event, since over the last 10 years no more than 45 cases per year have been reported and few community-acquired outbreaks have been described (12,13). In 2000, the number of reported cases in the Netherlands was 176, suggesting underdiagnosis in previous years.

No guidelines concerning the use and maintenance of whirlpool spa displays exist in the Netherlands. Our data demonstrate that contaminated spas may remain culture positive for months, perhaps as a result of stagnant water in their extensive inner tubing system. Because *Legionella* is ubiquitous in water systems, prevention of Legionnaires' disease depends mainly on disinfection. This study shows that whirlpool spas may become a health hazard if their disinfection system fails. Bathing in whirlpool spas has led mainly to outbreaks of Pontiac fever (14-18) and, to lesser extent, of Legionnaires' disease (6,19,20). Our data show that even staying in the vicinity of a whirlpool spa or walking in a hall where an operating whirlpool spa is on display may be important risk factors for Legionnaires' disease. Considering the popularity of whirlpool spas at home and the number of exhibitions where they are displayed, we suspect that small outbreaks have occurred without detection.

Clearly, strict regulations concerning the use, maintenance, and display of whirlpool spas are needed. The public at large should be informed as to the potential health hazards posed by whirlpools spas in public facilities and at home.

Unique in this outbreak was the circumscribed time of exposure for each individual patient to an identified source of *Legionella* infection. The finding that in 16% of cases the reported incubation period exceeded 10 days has major clinical and public health consequences. This finding contrasts with that of the Philadelphia outbreak, when only two cases had such long incubation times (16 and 26 days, respectively) (10).

In this outbreak investigation, a unique combination of three epidemiologic approaches allowed a comprehensive understanding of the chain of events, even as the investigators were confronted with numerous potential sources, three of which were culture positive. Our simple risk assessment of devices capable of spreading *Legionella*-infected aerosols proved to be an effective and timely predictor of the likelihood that a device was a source of the outbreak. The assessment and subsequent cultures revealed that the whirlpool spa in hall 3 was most likely the major source because it had been in continuous operation and its water had not been changed during the exhibition, unlike the whirlpool spa in hall 4. The bubblemats in halls 3 and 4 were demonstrated in room-temperature water, which was changed several times during the exhibition. Both mats had been dried and stored by the time of the environmental sampling.

The results of the case-control study indicate that pausing at this whirlpool spa was the most important consumer-related risk factor. Information bias related to this outcome is probably minimal, since the Dutch news media never mentioned the site of the whirlpool spa when reporting on the origin of the outbreak.

The results of the cohort study show that the average antibody levels were highest in the right side of hall 3, near the whirlpool spa. Plotting the geometric mean IgM and IgG titers of the nearest 35 exhibitors per surface area demonstrated that proximity to the whirlpool spa in hall 3 was associated with an increase in antibody titers, whereas this association was absent in hall 4. These results correlate with the inverse relation between attack rate among staff members and distance to the whirlpool spa in hall 3. The smoothing technique used in our

analysis gives an average antibody titer (for the nearest 35 exhibitors) per square meter. The exhibition hall was divided into 63-cm^2 squares, and for each square the smoothed average antibody titer was calculated and a color was assigned corresponding to a certain titer range. The color pattern gives an idea of the pattern of infected aerosols or movement of exhibitors.

The whirlpool spa in hall 3 had just been purchased; it was filled on February 17 and kept at 37°C throughout the exhibition. The concentration of *L. pneumophila* must have risen to levels infectious for immunocompromised visitors from February 21 onward and healthy visitors from February 23 onward. Similar growth rates have been reported (21,22). The increasing attack rate per day indicates that the continued growth of *Legionella* led to spread of aerosols bearing ever-increasing infectious doses. The lower attack rate on the last day of the exhibition may reflect the different composition of the visitor population on Sundays, when young families with children predominated, compared with weekdays, when elderly visitors predominated.

Although all cultures of specimens from the two separate parts of the local water supply system were negative, it is probable but unproven that the *Legionella* strains cultured from the whirlpool spas and the sprinkler installation originated from the local system. The finding of identical genotypes in these devices supports this hypothesis.

In conclusion, this large, severe outbreak in the Netherlands shows that diagnosis of *Legionella* pneumonia should lead to prompt investigation of the source of infection. Our comprehensive epidemiologic investigation identified a new whirlpool spa as the major source of the outbreak. Until strict regulations concerning the operation of whirlpool spas have been developed and issued, public exhibition of these devices in operation should be restricted.

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Address for correspondence: J.W. Den Boer, Municipal Health Service Zuid-Kennemerland, P.O. Box 1622, 2003 BR Haarlem, the Netherlands; fax: +31-23-5115970; e-mail: jwdenboer.ggdhaarlem@wxs.nl

Changes in Predominance and Diversity of Genomic Subtypes of *Bordetella pertussis* Isolated in the United States, 1935–1999

Terri Hawes Hardwick, Pamela Cassiday, Robbin S. Weyant, Kristine M. Bisgard, and Gary N. Sanden

Pulsed-field gel electrophoresis (PFGE) of *Bordetella pertussis* chromosomal DNA fragments generated by *Xba*I restriction has been used to subtype isolates for epidemiologic studies. To better understand the natural history of pertussis, we determined the PFGE profiles of 1,333 strains isolated in the United States from 1935 to 1999. Results showed a shift in prevalent profiles from the earliest to the latest study periods. In addition, genetic diversity decreased over time, and prevalent profiles were more highly related to each other than to less common profiles. These results provide the foundation for investigating the impact of prevention strategies, including the use of the acellular vaccines, on the currently circulating *B. pertussis* population.

Pertussis, or whooping cough, is an acute respiratory disease caused by *Bordetella pertussis*. Before pertussis vaccine was introduced in the United States in the mid-1940s, pertussis was a major cause of childhood illness and infant death (1). Pertussis incidence decreased after vaccination programs were introduced, and cases were reduced >90% compared with the prevaccination era. Despite vaccine intervention, pertussis remains endemic and epidemic peaks recur every 3 to 5 years (1-8). Since the early 1980s, the reported incidence of pertussis has increased, especially among adolescents and young adults (1,2,4).

Because vaccination coverage has been continuously high during this period, the observed shift in pertussis epidemiology may be a consequence of pertussis diagnosis and reporting or host factors, such as waning vaccine-induced immunity. A third explanation involves changes in the circulating *B. pertussis* population leading to increased virulence or resistance to vaccine-induced immunity. Differences in *B. pertussis* genetic subtypes circulating in the pre- and post-vaccination eras were demonstrated in the Netherlands, but the role of vaccination in subtype selection remains equivocal (9-11). Consequently, documentation of the natural history of the U.S. *B. pertussis* population may help explain recent and past changes in pertussis epidemiology and provides the comparative basis for recognizing current and future trends, including those potentially associated with prevention strategies.

Two principal technologies differentiate epidemiologically relevant strains of *B. pertussis*: gene-sequencing analysis and pulsed-field gel electrophoresis of genomic DNA fragments generated by endonuclease restriction (PFGE). In general,

sequencing analysis applies to genes with specified relevance, such as virulence determinants, while PFGE subtyping provides a broader perspective of the genome. PFGE subtyping has produced stable and highly reproducible profiles of *B. pertussis* isolates in different laboratories and has sensitively discriminated among epidemiologically distinct isolates (12-15). Therefore, we used genetic subtyping of recently and previously circulating *B. pertussis* isolates to elucidate the natural history of this pathogen in the United States from 1935 to 1999 and assessed subtypes for temporal trends.

Material and Methods

Bacterial Strains

A total of 1,333 *B. pertussis* strains were examined, including a convenience sample of 16 strains from 1935 to 1965 (designated archival); 127 strains from 1966 to 1985 (designated medial); and 1,190 strains from 1986 to 1999 (designated contemporary). Archival and medial isolates were from several different collections. Many of the later contemporary isolates were from enhanced surveillance programs. Overall, 61% of the isolates were from the metropolitan Cincinnati area (n = 480), Massachusetts (n = 205), or Minnesota (n = 125). A second analysis of PFGE profile results excluded the 480 Cincinnati isolates (1989 to 1996) and thus was based on 710 contemporary and 853 total strains. (A table listing all strains and PFGE profiles is available upon request.) Profiles were designated according to Centers for Disease Control and Prevention nomenclature, whereby: CY = *Bordetella*; X = *pertussis*; XI = *Xba*I; and three number fields identify the PFGE profile. Thus, CYXXI-010 identifies PFGE profile #10 derived from *Xba*I restriction of *B. pertussis* genomic DNA. *B. pertussis*

isolates were grown for 3 to 5 days at 37°C on charcoal agar supplemented with 10% defibrinated horse blood under ambient air and high humidity.

Preparation of Chromosomal DNA

Chromosomal DNA was prepared by following the PFGE protocol published by Gautom (16), with modifications. Cells of each strain were harvested after 3 to 5 days' incubation, and suspended in 2 mL of Tris EDTA buffer. The optical density of each cell suspension was adjusted between 0.48 and 0.52 by using a MicroScan turbidity meter (Baxter Diagnostics Inc., Deerfield, IL). Two hundred microliters of each suspension were transferred to 1.5-mL microcentrifuge tubes, and 10 µL of a 20 mg/mL solution of proteinase K (Amresco, Solon, OH) was added. Tubes were gently inverted six times, and 200 µL of the previously described 1.6% InCert agarose/SDS solution was added. The cell and agarose suspensions were mixed without aerosolizing by aspirating and expelling with a 200-µL pipetter and were immediately dispensed into two wells of a 100-µL disposable plug mold (Bio-Rad Laboratories, Hercules, CA) and allowed to solidify at ambient temperature. Plugs were transferred to 2-mL round-bottom microcentrifuge tubes containing 1.5 mL of the previously described EDTA/Sarcosine buffer and 40 µL of a 20-mg/mL solution of proteinase K. Tubes were immersed horizontally in a reciprocal shaking water bath (130 strokes per minute) at 50°C and incubated for 1.5 hours. The plugs were then transferred to 50-mL conical centrifuge tubes and washed with 10 mL of sterile distilled water for 15 minutes, then five times in 10 mL of the previously described Tris EDTA buffer for 30 minutes each. For each wash, tubes were placed in a reciprocal shaking water bath at 50°C and 150 strokes per minute. Plugs were stored and maintained in fresh plug wash buffer at 4°C for up to 4 months before restriction endonuclease digestion.

Restriction Endonuclease Digestion and PFGE

Two 1-mm slices of the prepared plugs were cut with a sterile razor blade and transferred to 1.5-mL microcentrifuge tubes containing 100 µL of *Xba*I digestion solution (30 U of *Xba*I in the manufacturer's supplied buffer; Roche Diagnostics, Indianapolis, IN). Plug slices were incubated at 37°C for 1.5 hours. One plug slice of each strain and three slices of a phage lambda molecular weight standard (PFGE Marker I; Roche Diagnostics) were placed on the teeth of a 15-well comb and allowed to adhere for 15 minute at ambient temperature. Molten 1% agarose in 0.5X Tris-Borate-EDTA buffer (TBE; Gibco BRL, Rockville, MD), cooled to 50°C, was poured and allowed to solidify around the comb. The comb was removed and the resulting wells were filled with agarose. PFGE was conducted in 0.5X TBE buffer in a contour-clamped homogenous electric field apparatus (CHEF-Mapper or CHEF-DR III; Bio-Rad). Electrophoresis was performed at 6V/cm for 18 hours at 14°C with a ramped switch time of 2.16 seconds to 35.07 seconds. Gels were stained for 25 minutes with gentle rotation in 250 mL of deionized water containing 25 µL of a 10

mg/mL solution of ethidium bromide. Unbound ethidium bromide was removed by washing gels in deionized water 3 times for 30 minutes each. DNA fragments were visualized on a UV transilluminator, and gels were photographed. The photographic negatives were scanned, and the DNA fragments were analyzed with Diversity Database software (Bio-Rad) with at least one band discrepancy as the basis for discriminating among profiles. *B. pertussis* DNA fragment sizes were determined from their motilities relative to the phage lambda molecular weight standard.

Statistical Analysis

We calculated genotypic diversity (GD) among profiles as follows: $GD=[n/(n-1)](1-\Sigma x_i^2)$, where x_i is the frequency of the ith PFGE profile and n is the total number of *B. pertussis* strains. Values of GD range from 0 to 1, with "0" defined as a population of isolates demonstrating identical profiles and "1" representing a unique profile for each isolate. The statistical significance of differences in GD values was evaluated by computer simulation as described previously (11). A PFGE profile relatedness diagram was constructed based on the unweighted pair-group method with arithmetic mean (UPGMA).

Results

We identified 105 PFGE profiles among the 1,333 *B. pertussis* isolates collected in the United States from 1935 to 1999 (Figure 1). We attempted to assess the geographic distributions of identified profiles in the United States. However, the geographic distribution of archival and medial isolates was limited by isolate collections available, which underrepresented certain regions. Thus, geographic trends in PFGE profile prevalences were not discernible.

We also evaluated the temporal distribution of isolate profiles (Figures 1 and 2). Eleven, 35, and 17 profiles were observed among 16 archival, 127 medial, and 1,190 contemporary isolates, respectively. Specific profiles tended to be restricted to isolates circulating during a given time period: only 5 (CYXXI-014, -020,-064, -071, and -074) of the 11 archival isolate profiles were also detected in strains circulating in the medial period; and 15 (CYXXI-003, -004, -006, -010, -014, -015, -017, -020, -021, -027, -053, -064, -066, -074, and -114) of the 35 medial profiles were shared by contemporary isolates. Moreover, only four PFGE profiles (CYXXI-014, -020, -064, and -074) were identified in 62 isolates cultured in all three time intervals (Figure 2). Profiles recovered from more than one time period tended to occur in low frequency in later periods. We observed only 4 of 16 frequencies >5% in a subsequent period: CYXXI-020 and CYXXI-064 were seen in 8% and 9% of medial isolates, respectively, and CYXXI-010 and CYXXI-027 were found in 37% and 6% of contemporary isolates, respectively.

The most frequent profiles overall (CYXXI-010, -002, -013, and -027) represented 33%, 10%, 9%, and 7%, of study isolates, respectively (Table). However, the most frequent pro-

Figure 1. Relatedness of pulsed-field gel electrophoresis profiles of *Bordetella pertussis* strains isolated in the United States, 1935 to 1999.

files in any given time period varied. The most frequent archival profiles, CYXXI-014 (19%) and CYXXI-064 (19%), collectively represented more than one third of the strains isolated before 1966. As noted previously, the CYXXI-064 profile continued into the medial period and was the third most prevalent profile after CYXXI-027 (13%) and CYXXI-062 (12%). The three most prevalent contemporary PFGE profiles (CYXXI-010, -002, and -013) represented approximately 58% of the strains isolated since 1986. Profile CYXXI-082 emerged in 1997 and has rapidly increased in frequency to account for 9% of the *B. pertussis* isolates from 1997 to 1999.

We evaluated the similarities between PFGE profiles of *B. pertussis* isolates and recognized two major relatedness clusters (clades) that were 58% similar (Figure 2). Seven additional PFGE profiles were notable for their divergence (<50% similar). The three profiles (CYXXI-054, CYXXI-059, CYXXI-060) representing the three earliest isolates from 1935 and 1939 were only 31% similar to Clades I and II. Interestingly, contemporary isolates yielded the remaining four divergent profiles (CYXXI-008, -033, -048, and -091). The eight nondivergent profiles from the archival isolates formed a Clade I subgroup with 68% similarity. PFGE profiles from the medial isolates were unevenly distributed in both clades: 60% of Clade I profiles and 16% of Clade II profiles represented 87%

and 13% of the medial isolates, respectively. The medial profiles in Clade I were more similar to each other (89% were 68% similar) than those in Clade II. Although contemporary profiles were highly divergent, most (68%) resided in Clade II and represented 78% of these isolates.

A 62% similarity subgroup of Clade II profiles included the three most frequent PFGE profiles during the contemporary period and overall (CYXXI-002, -010, and -013). The most frequent archival (CYXXI-014 and -064) and the most frequent medial (CYXXI-027 and -062) profiles shared a 74% similarity subgroup within Clade I. Two of these predominant profiles (CYXXI-014 and -064) represented isolates from all three time periods, as did profile CYXXI-020, which was also in this relatedness subgroup. The fourth persistent profile, CYXXI-074, was also in Clade I, but not within this 74% similarity subgroup.

The overall GD among the study isolates was 0.86. The GD was 0.94 for the archival strains and 0.94 for the medial strains. The GD decreased to 0.82 for contemporary strains. GD among isolates from the period before vaccine intervention, 1935 to 1946, was 1.00 and decreased to 0.91 for the remainder of the archival strains isolated from 1955 to 1965. Only the decrease in GD between the medial (GD = 0.94) and contemporary (GD = 0.82) periods was significant (p<0.001).

We analyzed PFGE profile data, excluding the 480 contemporary isolates from Cincinnati (6), to evaluate how a large source of isolates might affect our results (Table). With this exclusion, 710 contemporary and 853 total isolates were tested. Because 9 of 79 profiles representing contemporary isolates were unique to Cincinnati (CYXXI-005, -008, -009, -022, -023, -025, -030, -032, -035), a total of 96 PFGE profiles, including 70 from contemporary isolates, were included in the analysis.

PFGE Profiles	Archival	Medial	Contemporary
Unique	6	20	63
Recurrent	5	15	15
Total Profiles	11	35	78

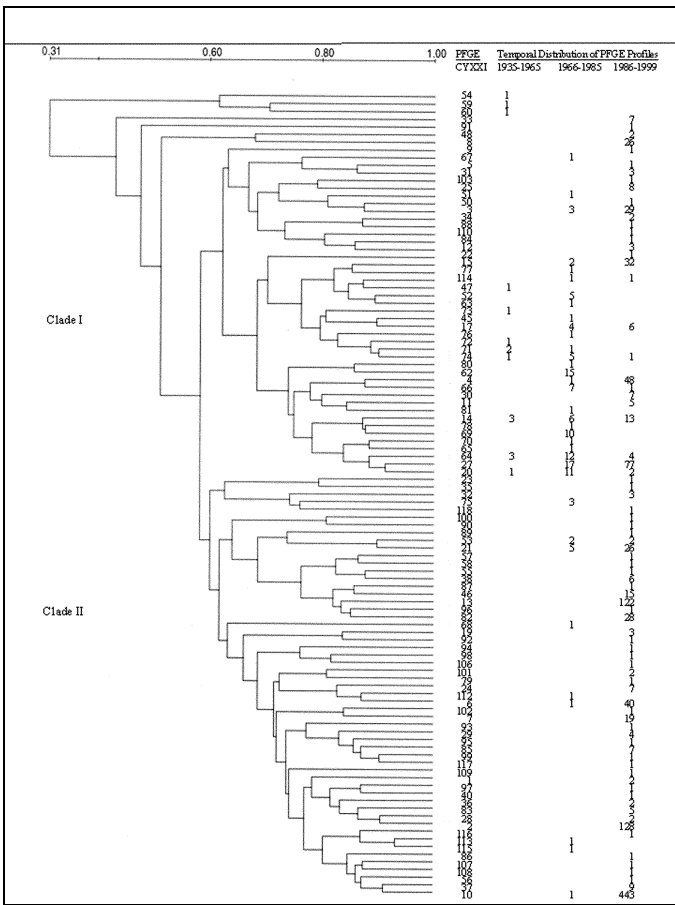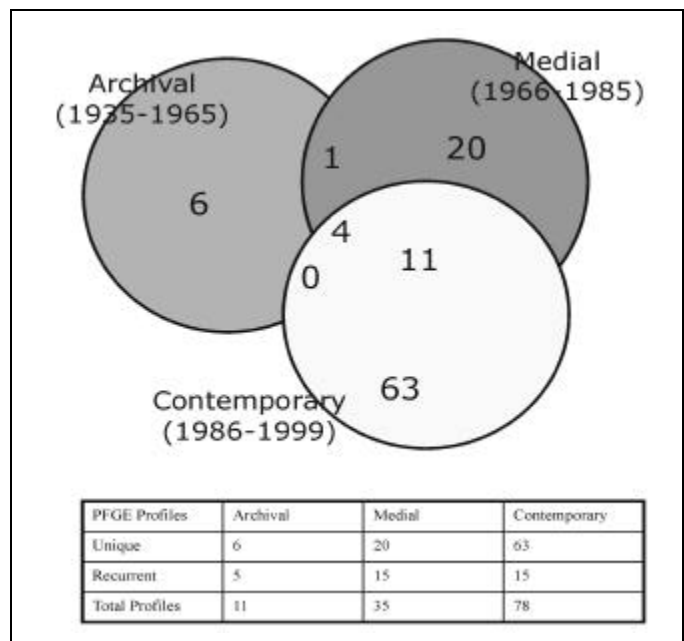Figure 2. Temporal distribution of 105 pulsed-field gel electrophoresis profiles of *Bordetella pertussis* strains isolated in the United States, 1935 to 1999.

Table. Frequencies of the most prevalent pulsed-field gel electrophoresis (PFGE) profiles of *Bordetella pertussis* strains isolated in the United States from 1935 to 1999

PFGE	Total US n=1,333	Total US w/o Cinn n=853	1935-1965 n=16	1966-1985 n=127	1986-1999 n=1,190	1986-1999 w/o Cinn n=710
2	9.6	4.3	-	-	10.8	5.4
3[a]	2.4	1.3	-	2.4	2.4	1.1
4[a]	3.7	0.4	-	0.8	4	0.3
6[a]	3.1	0.1	-	0.8	3.4	0.1
7	1.4	1.8	-	-	1.6	2.1
8	2	-	-	-	2.2	-
10[a]	33.3	33.9	-	0.8	37.3	40.8
13	9.2	14.1	-	-	10.3	17
14[b]	1.6	2.6	18.8	4.7	1.1	1.8
15[a]	2.6	0.8	-	1.6	2.7	0.8
17[a]	0.8	0.8	-	3.1	0.5	0.4
20[b]	1	1.5	6.3	8.7	0.2	0.1
21[a]	2.3	2.2	-	3.9	2.2	2
27[a]	7.1	9.3	-	13.4	6.5	8.8
37	0.7	1	-	-	0.8	1.3
46	1.1	1.8	-	-	1.3	2.2
52	0.4	0.6	-	3.9	-	-
53[a]	0.3	0.5	-	1.6	0.2	-
62	1.1	1.8	-	11.8	-	-
64[b]	1.4	2.2	18.8	9.4	0.3	0.6
66[a]	0.6	0.9	-	5.5	0.1	0.1
69	0.8	1.2	-	7.9	-	-
71[c]	0.2	0.4	12.5	0.8	-	-
74[b]	0.5	0.8	6.3	3.9	0.1	0.1
75	0.2	0.4	-	2.4	-	-
82	2.1	3.3	-	-	2.4	4
Total	89.5	88	62.7	87.4	90.4	89

[a]PFGE profile spans second and third time periods.
[b]PFGE profile spans all three time periods.
[c]PFGE profile spans first and second time periods.
CINN = Cincinnati, Ohio, data.

The prevalence of the predominant contemporary profile CYXXI-010 did not change from approximately 33%. Conversely, the overall prevalencies of two other predominant profiles were affected, as CYXXI-002 prevalence decreased from 10% to 4% and CYXXI-013 increased from 9% to 14%. Excluding Cincinnati isolates profiles, the total GD decreased slightly from 0.86 to 0.84, as did the GD for the contemporary isolates (1986 to 1999) from 0.82 to 0.79.

Discussion

There has been a resurgence of pertussis since the early 1980s in countries with high vaccination rates, including Australia (17), Canada (14), Denmark (18), the Netherlands (9,10,11,19), and the United States. Because this resurgence may represent changes in the etiologic agent, we determined the PFGE profiles of *B. pertussis* isolates circulating in the United States between 1939 and 1999 and evaluated these results for any trends with potential epidemiologic significance. We recognized several major trends: different profiles circulated and predominated at different times, the relatedness among the PFGE profiles was consistent with a relatively homogeneous population, the more frequent profiles were more highly related to each other than to less common profiles, and GD decreased over the study period.

The observed tendency of different profiles to circulate in different periods was also reported from the Netherlands, where 83% of DNA types were limited to single periods and only one type was cultured in all successive periods (11). Because PFGE profiles tended to be confined to a single time period, different profiles predominated in different periods. The two most predominant profiles (CYXXI-010 and -002) in the contemporary period emerged in the mid-1980s, temporally dissociated with changes in intervention programs such as vaccination, but coincident with an increasing incidence of pertussis (4). A shift in the frequency of allelic types of two immunogenic proteins, pertactin and pertussis toxin subunit A, was also noted during this period, and the new allelic types were strongly associated with isolates having the CYXXI-010 and -002 profiles (20). Additional study is needed to determine the role of the newly predominant allelic types in conferring predominance to these PFGE profiles, especially their distribution among less frequent profiles.

Our most dominant (33%) profile (CYXXI-010) is analogous to Canadian profile (a), which represented 34% of all Canadian isolates, suggesting that this profile is widely circulating in North America. We also noted that PFGE profile CYXXI-082 emerged in 1997 but still accounted for >2% of the contemporary isolates, confirming the dynamic nature of the population and suggesting that alternative profiles may predominant in the near future.

We determined PFGE profile similarities and evaluated them for potential associations, especially with predominance or persistence. No two of the 105 profiles were >93% similar, and two major similarity groups, or clades, were identified. The *B. pertussis* population studied in the Netherlands showed similar organization (11). This relatedness structure most likely represents the relatively homogeneous or clonal nature of most pathogenic bordetellae, as previously suggested by multilocus enzyme electrophoresis (21), DNA polymorphism studies (22), and earlier characterizations of strains by PFGE profiling (15,23). The distribution of several PFGE patterns over all study periods and multiple locations is also consistent with a homogeneous population. In general then, the observed *B. pertussis* population structure likely represents adaptation to a single host species and limited opportunity for horizontal genetic exchange, although such exchange between *B. pertussis* and *B. parapertussis* is evident (22,24).

The most prevalent profiles overall and in the median period tended to be relatively highly related to each other, suggesting that they may share common, but unknown, genetic

characteristics that confer dominance. The four persistent profiles that were observed in isolates from all time periods clustered within Clade I with 68% similarity, suggesting that they may share common properties for longitudinal transmission and that these properties are different and unlinked from elements conferring highest prevalence.

The similarity decreased between profiles from archival and successive periods, and the most predominant profile in the contemporary period showed the greatest divergence. In particular, the PFGE profiles from the prevaccine era (before 1946) isolates were highly divergent from the postvaccination isolate profiles. Others have proposed that the pre- versus post-vaccination era population changes may have been driven by selective pressure of whole-cell vaccines; the divergence in our study is consistent with this proposal (9-11). However, the role of vaccine selection in the divergence observed in our study remains speculative because a relatively low number of isolates was available from the archival period, GD did not decrease significantly until the contemporary period, and similarly divergent PFGE profiles were circulating in the contemporary period.

The divergence among profiles from contemporary isolates was comparatively high, although the GD was lowest for this period. A previous study indicated that increased recovery of isolates led to detection of PFGE subtypes circulating at low frequency (25). This suggests that the relatively large number of contemporary isolates permitted identification of infrequent, but divergent profiles not discernible in isolates from the other two periods. Despite this observed divergence and the diversity it implies, the frequency of predominant contemporary PFGE subtypes was sufficiently high to depress the GD relative to the previous periods.

The GD (0.86) of the *B. pertussis* population studied resulted from the prevalence of only a few PFGE profiles, combined with the low numbers of isolates represented by most of the profiles. Investigations in Canada (14), the Netherlands (11), Italy (26), and Mexico (27) showed a similar tendency: a few genomic profiles dominated and all others were observed infrequently. Published GD values were also similar in Dutch (11) and Mexican (27) studies, so our observations may be characteristic for *B. pertussis* populations.

GD was consistent between the archival and the medial isolates, but decreased significantly for the contemporary strains. This reflects relatively fewer profiles representing a greater proportion of contemporary isolates. In contrast, other investigators found a significant decrease in GD in the period after introduction of the whole-cell vaccine. Because the size of our archival time period may have precluded our ability to distinguish a temporally relevant shift in GD, we also calculated GD among isolates from early (1935 to 1955) and late (1956 to 1965) in this period. However, the change in GD was relatively modest, from 1.00 to 0.93.

Evaluating the population structure of *B. pertussis* requires sampling isolates representative of the natural distribution of circulating strains. Three sampling artifacts were inherent in our study. First, the numbers and geographic distribution of archival and medial isolates were limited by the isolate collections available so that the number of contemporary isolates was comparatively much greater. Second, most of the contemporary isolates were from an enhanced surveillance project comprising only six sites (in Arizona, Georgia, Illinois, Massachusetts, Minnesota, and New York). Consequently, the restricted geographic distribution of available isolates precluded evaluating our results for relevant geographic trends. However, the isolation of several prevalent profiles, such as CYXXI-010, from multiple sites in the United States and Canada was consistent with an essentially clonal population (21).

Third, an outbreak investigation such as occurred in Cincinnati from 1991 to 1996 and Delaware in 1986 can contribute a disproportionate number of isolates from a local area over a short time because they are more likely to be recovered and retained. To investigate the impact of this bias, we calculated results with and without the Cincinnati PFGE profile data and found that these results did change the prevalences of two of the three predominant profiles, but not GD. Thus, regional differences in profile frequencies apparently exist; future work will attempt to confirm this. However, the impact of epidemic strains did not seem to extend to the general population structure, as defined by GD. Epidemic isolates may limit PFGE profile diversity, especially if clonal expansion had a major role in causing the epidemic. However, we and others have shown that this is not the usual case with pertussis epidemics in general and in the Cincinnati epidemic in particular (11,14,24).

This study is the first to provide data about the distribution of PFGE profiles among the U.S. *B. pertussis* population over an extended period of time. Our results, together with pertussis surveillance data, can serve as the comparative basis for evaluating the potential impact of current and future prevention strategies, including the use of acellular vaccines, on the circulating *B. pertussis* population.

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Address for correspondence: Gary N. Sanden, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Mailstop D11, 1600 Clifton Road, Atlanta, GA 30333, USA; fax: 404-639-3023; e-mail: gns1@cdc.gov

Genetic Characterization of the M RNA Segment of *Crimean Congo Hemorrhagic Fever Virus* Strains, China

**Anna Papa,* Benjiang Ma,† Sophie Kouidou,* Qing Tang,† Changshou Hang,†
and Antonis Antoniadis***

We report the genetic characterization of the M RNA segment of *Crimean Congo hemorrhagic fever virus* (CCHFV). Two CCHFV strains isolated in Xinjiang Province, a region endemic for CCHF in northwestern China, were studied. These strains, designated BA66019 and BA8402, were isolated in 1965 and 1984 from a CCHF patient and *Hyalomma* ticks, respectively. Viral RNA was extracted from suckling mouse brains infected with these two strains, amplified, and sequenced. The full-length M RNA, consisting of 5.3 kb, was determined for both strains. The coding nucleotide sequences of the two strains differed from each other by 17.5% and from the reference CCHFV strain IbAr10200 by a mean of 22%, suggesting that the genus *Nairovirus* comprises a group of genetically highly diverse strains.

Crimean Congo hemorrhagic fever (CCHF), one of the most severe human viral diseases, has a death rate of up to 50%. CCHF is a public health problem in many regions of the world, including Africa, Middle East, southern and eastern Europe, and Western Asia (1-9). The causative agent, *Crimean Congo hemorrhagic fever virus* (CCHFV), is the type species of the genus *Nairovirus* in the *Bunyaviridae* family. The virus is transmitted to humans by the bite of *Ixodid* ticks (mostly of the *Hyalomma* genus) or by contact with blood or tissues from human patients or infected livestock. After an incubation period of 3 to 7 days, the patient has sudden onset of fever, chills, myalgia, headache, and rapidly evolving severe illness, followed by a hemorrhagic state with bleeding from the mucous membranes and petechiae, associated with thrombocytopenia and leukopenia (10). The highly pathogenic nature of the virus; the risk for spread from person to person through exposure to infected blood, respiratory secretions and excreta, resulting occasionally in serious nosocomial outbreaks; and the lack of an effective and safe therapy indicate the need for adequate guidelines for management of viral hemorrhagic fevers.

CCHFV, like all members of the genus, is a negative-stranded RNA virus with a tripartite genome consisting of a small (S), a medium (M), and a large (L) segment. The S RNA segment codes for the nucleocapsid (N) protein, and the M RNA segment codes for the glycoprotein precursor, resulting in the two envelope glycoproteins G1 and G2, while the L segment encodes the putative RNA-dependent polymerase. The first 8 to 13 nucleotide bases at the 3' ends of all three RNA segments have a sequence that is conserved in the viruses of this genus, with a complementary consensus sequence occurring at the 5' end; the ends of the segments are noncovalently linked so that the RNA occurs in a loosely bound circular configuration within the nucleocapsids (11). The M segment of nairoviruses is 30% to 50% larger than the M segments of members of other genera in the *Bunyavidae* family and has a potential coding capacity of up to 240 kDa of protein (12). CCHFV shares some common antigenic and genetic properties with *Dugbe virus* (DUGV), another member of the genus *Nairovirus*; some G1 epitopes may be conserved between the two viruses. Current knowledge of the molecular heterogeneity of CCHFV strains circulating in different parts of the world is very limited.

In China, the first CCHF cases were observed in 1965, when CCHFV strain BA66019 was isolated from a patient who lived in Xinjiang Province, an autonomous region in northwestern China which is the most CCHF-endemic area in the country. Another strain, BA8402, was isolated in 1984 from *Hyalomma asiaticum* ticks from the same region. The full-length S genome segment of these strains has been sequenced and analyzed (13). Over a 30-year period (1965 to 1994), 260 CCHF cases have been reported in China, 54 (21%) of them fatal. In 1997, an outbreak occurred in Honghai village (Bachu County, Xinjiang Province). During the 45-day outbreak period, 26 cases were reported, 5 of them fatal. Antibodies to CCHFV have been detected in humans and animals in the following provinces: Xinjiang, Qinghai, Sichuan, Yunnan, Anhui, Hennan, and Inner Mongolia (13).

Sequences of short S RNA CCHFV fragments from different parts of the world have shown considerable genetic differences (14-16). However, knowledge about the M RNA fragment is very limited, as no reports have been published and the only available complete M RNA sequence is that of the reference strain IbAr10200, isolated in 1970 from ticks in Nigeria (GenBank accession number U39455).

*Aristotelian University of Thessaloniki, Thessaloniki, Greece; and †Chinese Academy of Preventive Medicine, Beijing, China

The M gene is critical for immunity and pathogenicity, as well as for vaccine development. To define the molecular variability among CCHFV strains, we determined the nucleotide sequences of the M RNA genome segment of two CCHFV strains (BA66019 and BA8402) isolated in China. We also compared their predicted amino acid sequences with the respective sequences of the reference strain.

Virus Strains and Methods

Two CCHFV strains were studied. The first, strain BA66019, was isolated in 1965 from an ill resident of Xinjiang Province. The second strain, BA8402, was isolated in 1984 from *Hyalomma asiaticum* ticks from the same geographic region. Both strains were propagated in Vero E6 cells.

RNA was extracted from suckling mouse brains infected with the two strains by using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA was prepared with one gene-specific primer and random hexamers. We used 12 sets of primers to amplify 12 overlapping fragments of the whole M RNA of the two CCHFV strains. Primers were designed with the OLIGO primer analysis software (Molecular Biology Insights, Cascade, CO), first based on the known sequences of CCHFV strain IbAr10200 and later on the basis of the sequences determined. The amplified polymerase chain reaction (PCR) products were purified and sequenced in an ABI 373A Perkin Elmer fluorescent dye automated sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA). Sequences were aligned by ClustalW and genetic distances were estimated by the Kimura 2-parameter method with DNAdist program from the PHYLIP package (17). A phylogenetic tree was constructed by using Seqboot, DNAdist, Fitch and Consence from the same software package. Hydrophobicity plots were done by the Kyte and Doolittle method (18).

Results

Twelve overlapping M segment PCR products were amplified and sequenced in both directions, covering a continuous region of the whole M RNA segment of two CCHFV isolates, BA66019 and BA8402; the M RNA sequences were submitted to GenBank and assigned accession numbers AF350448 and 350449, respectively. The overlapping sequences were identical.

The complete M RNA genome of BA66019 was found to be 5,368 nt long, with a nucleotide composition of 30.9% adenine, 24.7% uracile, 22.1% guanine, and 22.3% cytocine (G/C content 44.4%). The 5' and 3' ends are perfectly complementary for 13 nt and partially complementary for 24 nt, with a mismatch at positions 14 and 16. A long open reading frame (ORF) is observed from the first methionine (AUG) start codon at nucleotide positions 78-80 to a stop codon at 5,142-5,144. A second in-frame initiation codon is at nucleotide position 93-95, in the same position as the start codon of the 10,200 strain. The ORF could encode a polypeptide of 1,689 amino acids with a predicted molecular weight of 186,952.8. The polypeptide sequence contains 12 potential asparagine-linked glycosy-

lation sites (Asp-X-Ser/Thr), and the theoretical pI is 7.99. A hydropathy plot of the M segment protein sequence shows at least six highly hydrophobic, potentially membrane-spanning regions (Figure 1).

The complete M RNA genome of BA8402 is 5367 nt long, with a nucleotide composition of 30.8% adenine, 25.2% uracile, 21.8% guanine, and 22.2% cytocine (G/C content 44.6%). The 5' and 3' ends are complementary and identical with the respective nucleotides of strain BA66019. A long ORF is observed from the first methionine start codon at 78-80 to a stop codon at nt 5,142-5,144. Similar to BA66019 strain, a second in-frame initiation codon is also seen at position 93-95. The ORF could encode a polypeptide of 1,689 amino acids, with a predicted molecular weight of 187,194.1. The polypeptide sequence contains 10 potential asparagine-linked glycosylation sites; the theoretical pI is 7.82. A hydropathy plot of the M segment protein sequence shows at least six highly hydrophobic, potentially membrane-spanning regions, similar to the plot of strain BA66019 (Figure 1).

Alignment of the M RNA sequences of the two Chinese CCHFV strains with that of the reference strain showed considerable variability among the three strains. Greater divergence was observed at the first 240 amino acids of the M genome. The nucleotide and amino acid differences between CCHFV strains in M RNA coding regions are as follows: BA66019 versus BA8402, 17.55% and 13.09%; BA66019 versus IbAr10200, 21.37% and 16.53%; and BA8402 versus IbAr10200, 22.57% and 17.04%, respectively.

Both Chinese strains have an extra proline (CCC for BA66019 and CCU for BA8402) at position 126, which is missing from strain IBAr10200. A phylogenetic tree was constructed; DUGV was used as outgroup (Figure 2).
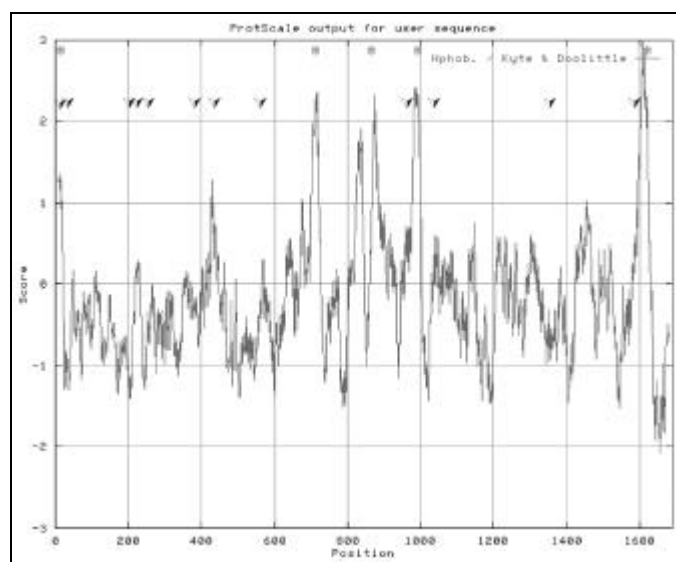
Figure 1. Hydropathy plot of the *Crimean Congo hemorrhagic fever virus* (CCHFV) strain BA66019 M segment open reading frame representing 1689 amino acids. Twelve potential N-linked glycosylation sites are indicated with an asterisk (*). The plot was constructed by the method of Kyte and Doolittle (1982) with a window size of 21. Hydrophobic residues appear above the line and hydrophilic residues below the line. Five strongly hydrophobic regions are marked (U̇).
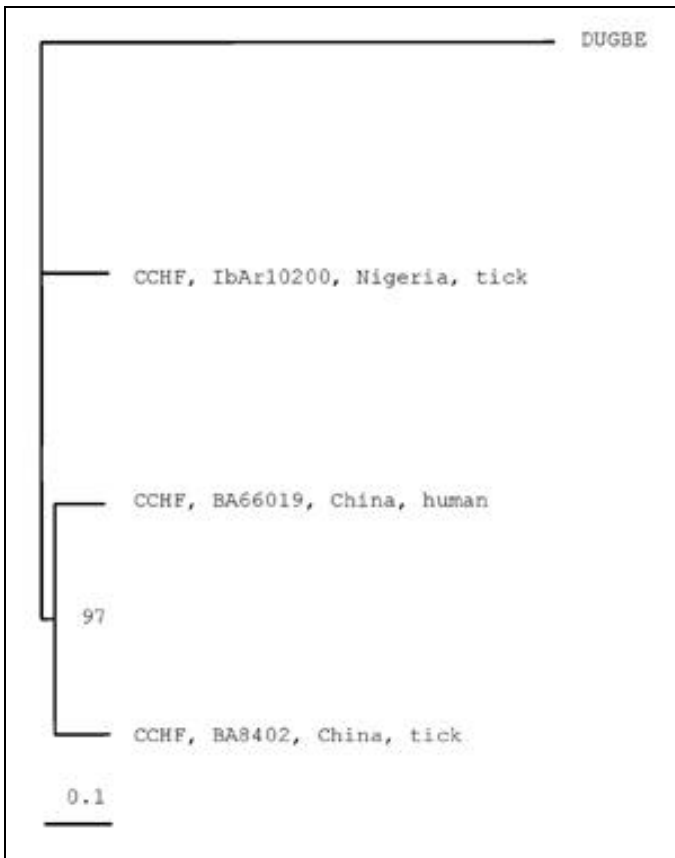
Figure 2. Phylogenetic tree based on 4,722 nt of the medium (M) RNA segment, including the two Chinese *Crimean Congo hemorrhagic fever virus* (CCHFV) strains BA66019 and BA8402 and the CCHFV strain IbAr10200, GenBank accession number U39455. *Dugbe virus* (DUGV) strain ArD44313, GenBank accession number M94133, was used as outgroup. Horizontal distances are proportional to nucleotide difference; vertical distances are for graphic display only. Bootstrap support (in %) is indicated at the respective branch.

Conclusion

The full-length M RNA sequences from two CCHFV strains were determined, representing the first published genetic characterization of CCHFV M RNA segment. The two strains were isolated in the region of China most endemic for CCHF.

Phylogenetic analysis of S RNA sequences showed that at least three subtypes of CCHFV N protein are in circulation, with substantial genetic variability among them, as a divergence of approximately 20% was observed among the three major clusters. One cluster contains sequences from Iran and West Africa; a second cluster contains viruses from locations throughout Africa, Asia, and the Middle East; and a third cluster contains a single virus from Greece (strain AP92). In addition, no obvious correlation with geographic distribution or time of isolation was seen (15,16). We were unable to construct an informative phylogenetic tree with M RNA sequences, as there are only three complete CCHFV M RNA sequences. However, in the constructed tree, the two Chinese strains cluster together with strong bootstrap support (97%), differing from the Nigerian tick isolate. The considerable difference

among the two Chinese strains and the IbAr10200 strain suggests that CCHF viruses comprise a genetically diverse group.

Prominent features of the M RNA segment are a high degree of divergence at the first part of the M genome, along with conservation of the middle and last regions and the 10-nt termini, which are conserved in all nairoviruses. In the S segment, a genetic difference of 3.3% was found between the Chinese BA66019 and BA8402 strains and a 15% difference between them and strain IbAr10200 (13). Estimation of the genetic distances of the M segment sequences after removal of the first 250 amino acids showed that the two Chinese strains differed from each other by 10% and from the IbAr10200 strain by 13%. The high degree of divergence of the first part of the genome (excluding the conserved termini) between the three strains indicates either that its function does not depend on a specific primary sequence, or, most probably, that the functional variability of these elements has no major impact on the CCHFV life cycle. Whether reassortment of RNA segments is a factor in CCHF epidemiology is not known.

The high degree of variability posed problems for the design of PCR and sequencing primers. First we designed primers based on the sequences of the reference strain, but new ones had to be designed on the basis of the Chinese sequences, as some differences in the genome led to annealing failure. Further M RNA sequences will help in determining the most conserved regions and subsequently the design of the most effective primers.

The nucleotide sequence and the coding strategy of the M RNA segment of DUGV have been determined (19). Comparison of the sequences of the three CCHFV and DUGV M segments showed that the M segment of DUGV is shorter than those of CCHFV. However, there is no evidence of truncation of the DUGV glycoprotein precursor, as observed at the N protein of DUGV. This N protein was found to be truncated relative to CCHFV and Hazara virus N proteins. This truncation may be a recent event in evolutionary terms, involving the mutation of an amino acid codon to a UGA stop codon (20). In estimating our results, we had in mind the first initiation codon, which is thought to have more favorable flanking sequences for initiation of protein synthesis, although use of leaky scanning mechanism could be the reason of initiation at the second AUG codon (21).

By analogy to other viruses of the *Bunyaviridae* family, the 1,689-amino acid product is supposed to be the precursor of the two glycoproteins, G1 and G2. Structural features may play a role in immunologic recognition of most important epitopes on the G1 and G2 proteins of these viruses. The positions of all 79 cysteine residues in both G1 and G2 proteins are conserved, suggesting that these two proteins are structurally similar. In addition, we observed that the glycosylation sites are conserved.

In conclusion, we determined the complete M RNA sequences of two CCHFV strains isolated in China. Additional complete genome sequences from human and tick CCHFV strains isolated in different parts of the world will help in iden-

tifying important conserved regions for the application of successful diagnostic methods, for the design of effective PCR and sequencing primers, and for the design of an effective vaccine. Analysis of such sequences will also elucidate the epidemiology of the virus, the exact phylogenetic relationship among different CCHFV strains, and subsequently the genetic evolution of the virus.

Dr. Papa is a senior scientist in the Department of Microbiology, School of Medicine, Aristotelian University of Thessaloniki, Greece. Her major interests are the molecular biology and epidemiology of nairo-, hanta-, and retroviruses.

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Address for correspondence: Anna Papa, Department of Microbiology School of Medicine, Aristotelian University of Thessaloniki, Thessaloniki, 54006 Greece; fax: 30-31-999-49; e-mail: annap@med.auth.gr

Prevalence and Genetic Profiling of Virulence Determinants of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolated from Cattle, Beef, and Humans, Calcutta, India

Asis Khan,* Shinji Yamasaki,*† Toshio Sato,† Thandavarayan Ramamurthy,* Amit Pal,* Simanti Datta,* Nandini Roy Chowdhury,* Suresh Chandra Das,‡ Asim Sikdar,‡ Teizo Tsukamoto,§ Sujit Kumar Bhattacharya,* Yoshifumi Takeda,¶ and Gopinath Balakrish Nair*#

We investigated the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) in hospitalized diarrhea patients in Calcutta, India, as well as in healthy domestic cattle and raw beef samples collected from the city's abattoir. Multiplex polymerase chain reaction using primers specific for *stx1* and *stx2* detected STEC in 18% of cow stool samples, 50% of raw beef samples, and 1.4% and 0.6% of bloody and watery stool samples, respectively, from hospitalized diarrhea patients. Various virulence genes in the STEC isolates indicated that *stx1* allele predominated. Plasmid-borne markers, namely, *hlyA, katP, espP*, and *etpD*, were also identified. Bead enzyme-linked immunosorbent assay and Vero cell assay were performed to detect and evaluate the cytotoxic effect of the Shiga toxins produced by the strains. STEC is not an important cause of diarrhea in India; however, its presence in domestic cattle and beef samples suggests that this enteropathogen may become a major public health problem in the future.

The first documented U.S. outbreak of diarrhea due to infection by Shiga toxin-producing *Escherichia coli* (STEC) belonging to the serotype O157:H7 occurred in 1982 (1). Since then, STEC has been increasingly recognized as an important human diarrheal pathogen and as the predominant cause of hemorrhagic colitis and hemolytic uremic syndrome (HUS). STEC is now being detected in 75% to 100% of episodes of sporadic HUS in Europe, North America, Canada, and Latin America, especially Argentina (2).

Multiple virulence factors contribute to the pathogenicity of STEC. Its main pathogenic property is the production of Shiga toxin (Stx), which inhibits the protein synthesis of host cells leading to cell death (3,4). *E. coli* Stxs are classified into two types, Stx1 and Stx2, and each type has several variants. Stx1 and Stx2 are encoded by alleles in the genome of temperate, lambdoid bacteriophages that remain integrated in the *E. coli* chromosome (5). The strains carry in their chromosome a 35-kb pathogenicity island called locus of enterocyte effacement (LEE), whose genes are responsible for generation of attachment and effacement lesions. LEE encodes a type III

secretion system, and some LEE genes are necessary for initiation of signal transduction events (6). However, not all STEC strains harbor the LEE, and it is not necessary to cause human infection. Moreover, STEC strains almost invariably harbor a 97-kb plasmid encoding possible additional virulence traits such as STEC hemolysin (which acts as a pore-forming cytolysin on eukaryotic cells [7]); the bifunctional catalase peroxidase KatP (8); a secreted serine protease (espP, which can cleave human coagulation factor V [9]); and the *etpD* gene cluster (which probably encodes a type II secretion pathway [10,11]).

Cattle and sheep are the primary reservoirs of STEC, but STEC has been isolated from deer, horses, dogs, and birds (12). Feces from any of these animals could serve as primary source for STEC. The dynamics of these pathogens in animals and environment is not well understood. Principally, STEC is transmitted through the consumption of contaminated foods such as raw or undercooked ground meat products and raw milk (13). Although cattle are the primary known reservoirs of STEC, humans may acquire STEC infections from other sources, possibly vegetables (14), fruit juice (15), or contaminated drinking water (16), or through direct contact with feces of infected persons (17).

In industrialized countries such as the United States, Japan, Germany, Australia, and United Kingdom, large outbreaks and many sporadic cases of STEC infections have been reported and have become a major health concern. More than 50 sero-

*National Institute of Cholera and Enteric Diseases, Calcutta, India; †Research Institute, International Medical Center of Japan, Shinjuku-ku, Tokyo, Japan; ‡Indian Veterinary Research Institute, Belgachia, Calcutta, India; §Osaka Prefectural Public Health Institute, Osaka, Japan; ¶National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan; and #International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh

types of STEC have been isolated from stool samples of patients with hemorrhagic colitis or HUS; however, many of these serotypes have not been as thoroughly or systematically characterized for virulence genes and properties as *Escherichia coli* O157:H7. To date, very few isolates of STEC from humans or animals have been reported in industrialized countries.

In India, there is a paucity of information on STEC. It has not been identified as an etiologic agent of diarrhea in India. Though a few strains of O157 serogroup have been isolated from sporadic cases of diarrhea, these strains have not been well characterized, and their origin is uncertain (18).

Serotyping of STEC alone is insufficient to assess the pathogenic properties of the strains because such organisms are quite variable in their repertoire of virulence determinants. Analysis of the genotypes of the STEC strains by the use of specific gene probes or polymerase chain reaction (PCR) provides more detailed information about genetic variability and subtypes (11).

In this study, we investigated the prevalence of STEC in healthy domestic cattle of a semi-urban community, in raw beef samples, and in hospitalized patients with diarrhea in Calcutta, India. Isolated STEC strains were characterized in detail to identify the predominant virulence genes and to understand how the Indian strains compare with STEC strains isolated elsewhere in the world.

Materials and Methods

Hospital Surveillance

Stool samples were collected from diarrheal patients enrolled in an ongoing active surveillance system at the Infectious Diseases Hospital (IDH) and from all the cases attending the B.C. Roy Hospital for Children, Calcutta, during a 1-year study period from January to December 1999. In the surveillance system, every fifth patient attending the hospital was included for sampling. Stool samples were collected in sterile McCartney bottles by using sterile catheters; sterile cotton-tipped swabs were used to take rectal swabs from patients from whom stool could not be obtained. Rectal swabs were placed in Cary-Blair medium, and stool samples were transported to the laboratory within 1 hour of collection. All samples were examined for STEC and other enteric pathogens such as other diarrheogenic *E. coli*, vibrios, *Salmonella* spp, *Shigella* spp, *Aeromonas* spp, rotavirus, parasites, and protozoans, following standard methods (19).

Cow Stool Samples

One hundred forty stool samples were collected from domestic cows of a semi-urban community near Calcutta; 66 stool samples were obtained from the state livestock farm at Kalyani, 71 kms away from Calcutta. These samples were collected at no defined periodicity during the year 1999.

Beef Samples

A total of 111 beef samples were collected from Calcutta Municipal Corporation abattoir, situated in Tangra. Collections were made twice a month during March to July 1999, the season when the maximum number of diarrhea patients is admitted to IDH and other Calcutta hospitals.

Enrichment of Samples

A loopful of human or cow stool samples was directly inoculated into 3 mL of Bacto EC Medium (Difco, MI, USA) for enrichment and incubated overnight at 37°C under shaking conditions. With beef samples, 50 mL of EC broth was aseptically transferred into a polythene bag and mixed well with the beef sample. After 2 hours, the broth was transferred into a sterile conical flask and incubated overnight at 37°C with constant shaking. After overnight incubation, STEC was screened by using a variety of screening methods as described below.

Screening Strategies

Enrichment Broth PCR

After incubation, enriched broth was directly examined by PCR using *stx1* and *stx2* primers under the conditions described in Table 1. Broth cultures that yielded positive PCR results for either *stx1* or *stx2* or both were serially diluted in 10 mM phosphate-buffered saline (PBS) (pH 7.0) and 100 µL volume of each dilution was spread on Luria agar (Difco) plate in duplicate. Randomly picked colonies were further screened for the presence of STEC by PCR or colony hybridization using DIG-labeled *stx1* and *stx2* gene probes as described below.

PCR Screening for Single-Cell Isolation

Single colonies were randomly picked from the dilution plates and spot inoculated on a master Luria agar plate and then inoculated in 3 mL EC broth. Four colonies were pooled for each EC broth and incubated overnight at 37°C in a shaker. Attempts were made to inoculate as many colonies as possible from each plate. Following overnight incubation, the inoculated EC broth culture was diluted 10-fold with sterile PBS and boiled for 10 minutes. This method was used as the template for a multiplex PCR using primers for *stx1* or *stx2* genes (Table 1). PCR was done as described previously (20). When a positive PCR result was obtained, the PCR was repeated to determine which of the four pooled colonies contributed to the positive result. Once an STEC strain was identified, it was preserved in Luria broth supplemented with 15% glycerol at -70°C.

Colony Hybridization for Single-Cell Isolation

An alternative approach adopted to identify STEC colonies from the dilution plates was the colony hybridization procedure, followed as described previously (11,23). Positive STEC colonies, if present, appeared as dark purple spots on the membrane. Colonies in the dilution plates were matched with the

Table 1. Polymerase chain reaction (PCR) primers and conditions used in this study

Primer no.	Nucleotide sequence of primers	Target	PCR conditions[a]			Amplicon (bp)	References
			Denaturing	Annealing	Extension		
EVT1/ EVT2	5'-CAACACTGGATGATCTCAG-3' 5'-CCCCCTCAACTGCTAATA-3'	*Stx1* family	94°C, 60s	55°C, 60s[b]	72°C, 60s	349	20
EVS1/ EVC2	5'-ATCAGTCGTCACTCACTGGT-3' 5'-CTGCTGTCACAGTGACAAA-3'	*Stx2* family	94°C, 60s	55°C, 60s[b]	72°C, 60s	110	20
hlyA1/ hlyA4	5'-GGTGCAGCAGAAAAAGTTGTAG-3' 5'-TCTCGCCTGATAGTGTTTGGTA-3'	EHEC *hlyA*	94°C, 30s	57°C, 60s	72°C, 90s	1,551	7
wkat-B/ wkat-F	5'-CTTCCTGTTCTGATTCTTCTGG-3' 5'-AACTTATTTCTCGCATCATCC-3'	katP	94°C, 30s	56°C, 60s	72°C, 15s	2,125	9
D1/ D13R	5'-CGTCAGGAGGATGTTCAG-3' 5'-CGACTGCACCTGTTCCTGATTA-3'	etpD	94°C, 30s	52°C, 60s	72°C, 70s	1,062	21
EAE1/ EAE2	5'-AAACAGGTGAAACTGTTGCC-3' 5'-CTCTGCAGATTAACCTCTGC-3'	eae	94°C, 60s	55°C, 90s	72°C, 90s	350	22

[a] Unless stated, PCR was done for 30 cycles.

[b] After 35 cycles, final extension step of 10 min at 72°C was performed.

spots on the membrane, and isolated STEC strains were reconfirmed by PCR, as described. Probes used included fragments of *stx1* (905 bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN501) (24) and *stx2*-A (860 bp *Bam*HI and *Eco*RI digest from recombinant plasmid pKTN502) (25). These probes were also used for the dot blot assay, which employed PCR amplicons obtained from enrichment cultures of human stool, cow stool, and beef samples to confirm the PCR specificity.

Screening for Pathogenic Factors by PCR

PCR for detecting both chromosomal and plasmid virulence markers was performed by using a thermal cycler in a total volume of 20 µL containing 2.5 mM of each deoxynucleoside triphosphate, 30 FM of each primer, 2 µL of 10X PCR buffer, and 1 U of r-Taq DNA polymerase (both from Takara, Shuzo, Otsu, Japan). Primer sequences and PCR conditions are given in Table 1.

Screening for Pathogenic Factors by Colony Hybridization

A colony hybridization test for detecting pathogenic factors was carried out as described previously (26). DNA probes for

espP, *katP*, *eae*, and *hlyA* were prepared by PCR. Primer sequences and PCR conditions are given in Table 2. After PCR, the products were purified by QIA Quick PCR Purification Kit (QIAGEN GmbH, Germany) in accordance with the manufacturer's instructions. The DNA probes were labeled by random priming method using Multiprime DNA labeling system and [α^{32}P] dCTP, and the colony hybridization procedure was followed as described (23).

Serotyping

Serotypes of the STEC strains were determined by slide agglutination with either commercially available O (poly and monovalent antisera) and H (monovalent antisera) (Denka Seiken Co., Japan) or antisera prepared at the Osaka Prefectural Public Health Institute, Osaka, Japan.

Vero Cell Assay

Preparation of Cell-Free Culture Filtrates

STEC strains were cultured in L-broth (Difco), at 37°C overnight with constant shaking. Bacterial cells were pelleted

Table 2. Polymerase chain reaction (PCR) primers and conditions for preparing DNA probes used in the colony hybridization test

Nucleotide sequence of primers	Target	PCR conditions[a]			Amplican (bp)
		Denaturing	Annealing	Extension	
5-GAGAATTCACAGATGGATATTTCAAATTTC-3 5-TCTCCTCGAGCTAGTTGACCTCGTTCAGAAACG-3	*espP*	94°C, 10s	68°C, 30s	72°C, 90s	2,900
5-GAGAGAATTCTTCCTGTTCTGATTCTTCTG-3 5-TCTCCTCGAGTCAAAACTTATTTCTCGCATCATCATCC-3	*katP*	94°C, 10s	68°C, 30s	72°C, 60s	2,130
5-TCTCAAGCTTTTAGAAATAGTCTCGCCAGTATTC-3 5-GAGAGAATTCGTCAGGAGGATGTTCAGG-3	*eae*	94°C, 10s	68°C, 30s	72°C, 90s	880
5-GAGAGGATCCGGTGCAGCAGAAAAAGTTGTA-3 5-TCTCCTCGAGTCATCTCGCCTGATAGTGTTTGG-3	*hlyA*	94°C, 10s	68°C, 30s	72°C, 60s	1,560

[a] PCR was done for 30 cycles.

by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was filter-sterilized by using 0.22-µm filters (Millipore, USA). The culture pellet was resuspended in PBS and then sonicated by using a Handy sonic (Tomy Seiko Co, Ltd., Tokyo, Japan), which was again centrifuged to remove debris. Supernatant was filter-sterilized. Both the culture supernatant and cell lysate were used for the assay.

Cytotoxic Assay

The cytotoxic effect of STEC strains was assayed on Vero cells in 96-well flat-bottom tissue culture plates (NUNC, Intermade, Denmark), as previously described (24). The cells were observed microscopically for 72 hours and the cytotoxicity titers determined; the highest toxin dilution that caused lysis of 50% of the cell monolayer was taken as the titer.

Bead ELISA

The initial procedure for preparation of cell-free culture supernatant and cell lysate was the same as for the Vero cell assay. The procedure for the bead ELISA has been described (23).

Hemolysin Activity

Hemolytic activity of the STEC strains was investigated by streaking the strains on tryptic soy agar (Difco) plates containing 5% washed and unwashed O group human blood cells. The hemolytic activity was observed from 3 to 18 hrs of incubation at 37°C (11).

Results

Prevalence of STEC in Human Stool Samples

The age distribution of the 1,241 and 284 patients whose samples were examined from IDH and B.C. Roy Hospital for Children, respectively, is shown in Table 3. Of the specimens from IDH patients during the period of surveillance, eight were positive for STEC by PCR in the preliminary screening. However, only nine strains could be isolated from six of the eight PCR-positive samples. The strains AK-11 and AK-26 were isolated from the same stool sample; AK-27, AK-28, and AK-29 were isolated from another single stool sample. Four of the 284 bloody stool samples examined from B.C. Roy Memorial Hospital for Children were positive for STEC by PCR, but again STEC could be isolated from only 3 of the 4 PCR-positive samples. Of note, 7 (58%) of 12 diarrhea patients from whom STEC was isolated were also co-infected with other pathogens (Table 4). STEC was isolated as the sole pathogen from 5 (42%) of 12 patients with diarrhea. The relative frequency of other enteric pathogens detected in the study samples is shown in Table 5.

Prevalence of STEC in Beef Samples

Of the 111 raw beef samples examined, 55 samples were positive for STEC when examined by multiplex PCR for *stx1* or *stx2*. However, STEC was isolated from only 4 of the 55

Table 3. Age distribution of patients admitted to hospitals where stool samples were examined

Age (in years)	Infectious Diseases Hospital (%)	B. C. Roy Hospital for Children (%)
≤ 5	25.1	97.6
>5-15	10.1	2.4
>15-25	20.2	-
>25-35	18.0	-
>35-45	11.0	-
>45-55	7.6	-
>55	8.1	-

PCR-positive samples by using the techniques adopted in this study.

Prevalence of STEC in Cow Stool Samples

Eight of 140 stool samples from cows examined from the community were positive for STEC; 10 STEC strains were recovered from the 8 samples. In contrast, of the 66 cow stool samples collected from Kalyani farm, STEC was detected in 29 by PCR. Only 5 strains could be isolated from the 29 PCR-positive samples.

STEC Serotypes

Of the 30 STEC strains isolated in this study, 8 strains were O antigen untypable with somatic (O) antiserum. However, except for one strain (AK-32), all the O-untypable strains were typable with different H-types (Table 6). Of the strains typed, four belonged to serotypes that are not listed in the updated list of serotypes of non-O157 STEC isolated from humans worldwide (http://www.microbionet.com.au/frames/feature/vtec/brief01.html). These are O96:H19, ONT:NM, ONT:H18, and ONT:H14. There was no clustering of any particular serotype. The serotypes ONT:H19 and O159:H12 were isolated from both humans and cows; otherwise, there was no match between serotypes of STEC isolated from human, cow, and beef samples (Table 6).

Analysis of Chromosomal Markers

stx1 and *stx2*

In our study, 30 strains were positive for *stx* by PCR. Of the 12 human isolates, 7 were positive for only *stx1*, 2 for only *stx2*, and 3 for both *stx1* and *stx2* (Table 6). Of the 14 strains isolated from cow stool samples, 6 carried *stx1*; 3, *stx2*; and 5 were positive for both *stx1* and *stx2*. Three of the four strains isolated from raw beef harbored *stx1* and *stx2*; the fourth carried only *stx1*.

eae

As in the case of enteropathogenic *E. coli* (EPEC), the characteristic attaching and effacing (A/E) ability is encoded by 41 genes present on the LEE pathogenicity island (6,27).

Table 4. Clinical manifestation and other information on patients from whom Shiga toxin-producing *Escherichia coli* was isolated

STEC strain number	Stool characteristics	Age (years)	Sex	Religion	Vomiting	Fever	Dehydration	Other pathogen(s) present in the stool samples
					Clinical manifestation			
AK-11[a] AK-26[a]	Watery	32	M	H	Negative	Negative	Severe	*Vibrio parahaemolyticus*
None	Watery	28	F	MU	Positive	Positive	No	None
None	Watery	17	F	H	Positive	Positive	Moderate	None
AK-18	Watery	30	M	H	Positive	Positive	Severe	*V. cholerae* O139
AK-27[a] AK-28[a] AK-29[a]	Watery	50	M	O	Positive	Positive	Severe	*V. cholerae* O1
AK-31	Watery	2	M	H	Positive	Negative	Severe	None
AK-32	Watery	40	F	O	Negative	Negative	Moderate	*V. cholerae* non-O1 non-O139
AK-33	Watery	27	M	MU	Positive	Positive	No	None
AK-14	Bloody	2½	M	MU	Negative	Positive	No	*V. parahaemolyticus, Shigella* spp.
AK-30	Bloody	2	F	MU	Negative	Positive	No	None
AK-40	Bloody	1½	F	MU	Negative	Positive	Moderate	*Shigella* spp.

[a] Strains isolated from one stool specimen but belonging to different serotypes.
H, Hindu; MU, Muslim; O, Other religion; M, male; F, female.

Pathogenicity of STEC is associated with the presence of LEE and in particular *eae*. We performed PCR with primers EAE-1 and EAE-2. An 863-bp PCR product was demonstrated for four strains (P-33-2-26, AK-16, AK-38, and AK-40), indicating the presence of *eae* (Table 6).

Analysis of Plasmid-Encoded Markers

E-hly, etpD, katP, and *espP* are plasmid-encoded markers. PCR with the hemolysin primers hlyA1 and hlyA4 showed that 12 of the 30 STEC isolates contained *E-hly* sequences. However, AK-1, although negative by PCR, hybridized with *hlyA*

Table 5. Other enteropathogens isolated from stool samples examined for Shiga toxin-producing *Escherichia coli*, India

Enteropathogens		Infectious Diseases Hospital (%)	B. C. Roy Hospital for Children
Vibrio cholerae	O1	9.5	0.6
	O139	5.2	-
	Non-O1 non-O139	3.7	3.1
Vibrio parahaemolyticus		2.8	0.4
Shigella	*flexneri*	0.4	0.7
	sonnei	0.1	-
	boydii	0.2	-
Salmonella		0.5	1.1
E. coli	EPEC	0.5	-
	ETEC	1.3	-

EPEC, enteropathogenic *Escherichia coli*; ETEC, enterotoxigenic *E. coli*.

probe (Table 6). The remaining strains were negative. However, P-33-2-26 and AK-38 produced a PCR product of 1062 bp, and primers D1 and D13R suggested the presence of *etpD* gene cluster. Seven strains possessed the *katP* gene cluster, as demonstrated by PCR with primers pair wkatB and wkatF as well as by colony hybridization. While *espP* could be detected in 11 strains when analyzed by probe complementary to *espP* sequence, only 2 strains generated the specific amplicon when PCR was carried out with *espP-specific* primer.

Thus, the analysis of virulence markers revealed that in the present collection of strains AK-38, a cow stool isolate not belonging to the O157 serogroup carried all the potential virulence genes.

Phenotypic Characterization of STEC Strains

Vero Cell Assay

Expression of Stx was examined by Vero cell cytotoxic assay. Of the 30 strains examined, all were cytotoxic to the Vero cells. AK-38, despite having *stx1* and the full repertoire of virulence genes, did not exhibit the cytotoxic effect (Table 6).

Bead ELISA

A highly sensitive bead-ELISA was applied to detect the presence of Stx1 and Stx2 in the cell-free culture filtrate and cell lysate of the isolates in this study. This assay identified Stx1 in 12 of 14 *stx1* PCR-positive strains but identified only 3 of the 5 *stx2* PCR-positive strains in both cell filtrate and lysate. Of the 11 strains positive for *stx1* and *stx2* by PCR, 8 were positive by Bead-ELISA for both toxins (Table 6).

Table 6. Serotype, phenotypic and genotypic traits of Shiga toxin-producing *Escherichia coli* strains, India

Strain no.	Serotype	Origin	Genotype (PCR/colony hybridization)							Phenotype				
			Chromosomal genes				Plasmid genes							
			stx1	stx2	eae	hlyA	etpD	katP	espP	Hemolysis	SF[a]	Vero[b]	Stx1	Stx2
AK-40	O11:H8	Human	+/+	-/-	+/+	+/+	-/-	+/+	-/+	E-hly	+	ND	ND	ND
AK-11	ONT:H18	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	-
AK-18	O156:H7	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-27	ONT:H19	Human	+/+	-/-	-/-	-/-	-/-	+/+	-/-	-	+	+	+	-
AK-30	O7:H6	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-32	ONT:NM	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	ND	ND	ND	ND
AK-33	O159:H12	Human	+/+	-/-	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	-
AK-26	O159:H9	Human	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-	+	+	-	+
AK-29	O110:H16	Human	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-	+	+	-	+
AK-14	ONT:H14	Human	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
AK-28	O43:H2	Human	+/+	+/+	-/-	+/+	-/-	-/-	-/-	-	+	+	+	+
AK-31	O96:H19	Human	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-	ND	ND	ND	ND
P-33-2-26	ONT:H2	Cow	+/+	-/-	+/+	+/+	+/+	+/+	-/-	E-hly	+	+	+	-
AK-38	O103:H2	Cow	+/+	-/-	+/+	+/+	+/+	+/+	-/-	-	+	-	-	-
P-179-101	O146:H1	Cow	+/+	-/-	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	-
C-45-3-19	O149:HNT	Cow	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-35	O22:H16	Cow	+/+	-/-	-/-	-/-	-/-	+/+	-/-	-	+	+	+	-
AK-39	O9B:H5	Cow	+/+	-/-	-/-	-/-	-/-	-/-	+/+	E-hly	+	+	+	-
C-5-1-13	O82:H8	Cow	-/-	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	-	+
AK-1	O2:H25	Cow	-/-	+/+	-/-	-/+	-/-	+/+	-/-	-	+	+	-	-
AK-37	O22:NM	Cow	-/-	+/+	-/-	+/+	-/-	-/-	+/+	-	ND	ND	ND	ND
C-2-1-15	O28ac:H21	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
C-3-2-25	ONT:H34	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
C-3-3-42-6	ONT:H19	Cow	+/+	+/+	-/-	-/-	-/-	-/-	-/+	E-hly	+	+	+	+
P-33-2-4	O88:HNT	Cow	+/+	+/+	-/-	+/+	-/-	-/-	-/+	E-hly	+	+	+	+
AK-36	O159:H12	Cow	+/+	+/+	-/-	-/-	-/-	-/-	-/-	E-hly	+	+	+	+
AK-16	ONT:H21	Beef	+/+	-/-	+/-	-/-	-/-	-/-	-/-	-	+	+	+	-
AK-12	O20:H12	Beef	+/+	+/+	-/-	-/-	-/-	-/-	-/-	ND	+	+	+	-
AK-13	O110:HNT	Beef	+/+	+/+	-/-	-/-	-/-	-/-	-/-	ND	+	+	+	-
AK-17	O172:NM	Beef	+/+	+/+	-/-	+/+	-/-	+/+	-/+	E-hly	+	+	+	+

[a]Fermentation of sorbitol.

[b]Vero cell cytotoxicity.

NT, Not typable; ND, Not determined; NM, nonmotile.

Hemolysin

Fourteen of the thirty STEC isolates showed a hemolytic phenotype that resembled alpha-hemolytic activity rather than the typical enterohemolytic phenotype (Table 6). The zone was clear, large, and could be visualized after 3 to 18 hours' incubation at 37°C. Of the 14 strains, 9 were positive by PCR using E-*hlyA* primer pair. However, three other STEC, which carried the *hlyA* gene, did not produce hemolysin.

Discussion

During the past decade, STEC has evolved from a clinical novelty to a global public health concern. STEC infections have been reported from over 30 countries on six continents, causing a spectrum of human illness ranging from symptom-free carriage to severe bloody diarrhea and even to life-threatening sequelae such as HUS. However, there is a paucity of reports on STEC in the developing world including India. A

previous study in India found no STEC in children with diarrhea in Delhi (28); a study from Bangladesh reported that no STEC were recovered from children with diarrhea (29). This is possibly related to lack of surveillance for this organism because of the difficulty in isolating STEC. However, in an outbreak of bloody diarrhea in Cameroon, half the patient specimens yielded STEC and half *Shigella* species (30).

We attempted to document the prevalence of STEC in Calcutta and provide information on the phenotypic traits, serotypes, and molecular characterization of the virulence genes. The reasons for the low prevalence of STEC-associated diarrhea in Calcutta and possibly other places in India are not well understood. Perhaps Indians acquire protective antibodies at an early age or use cooking practices that effectively eliminate STEC.

The isolation rate of STEC from hospitalized secretory and bloody diarrhea cases was very low in this study, and there was no evidence of a seasonal distribution of STEC-positive samples. Among STEC-identified diarrhea cases, 58% were found to be infected with other enteric pathogens. One case was positive for *Vibrio parahaemolyticus*, STEC, and *Shigella*. In such situations, it is difficult to conclude the role played by STEC in causing disease. We included religion in the data analysis since the food habits of Hindus and Muslims vary (e.g., Muslims eat beef) (Table 3). Indirect routes of transmission may, however, be more significant in India, where most of the population does not eat beef for religious reasons. Possibly in areas like this, STEC transmission could occur through exposure to vegetables, fruits, or drinking water contaminated by bovine feces or through direct contact with feces of infected persons (14-17). Only non-O157 STEC strains were isolated from human cases. In general, with the exception of North America and Japan, non-O157 STEC strains are isolated more frequently than O157, with a median of a fourfold higher isolation rate but with wide variation among studies (31). From our intense survey conducted over a 1-year period, it was clear that STEC are not currently a major diarrhea-causing etiologic agent in India. However, this study indicates that strains of *E. coli* with the O157 lipopolysaccharide and non-O157 LPS are present in the milieu, as are virulence genes, which are known to contribute to STEC virulence. Mixing and matching of genes in the environment or in the human intestine could lead to the evolution of pathogenic STEC.

The prevalence of STEC in bovine fecal flora and beef was high, according to PCR results. The occurrence of STEC in raw beef samples strongly indicates that unhygienic practices prevailed in slaughterhouses in Calcutta. Therefore, STECs are clearly present in beef and, as in other countries (12), there is a bovine reservoir of STEC in India. Isolation of twelve O157:H7 strains from 9 of 25 beef samples originally imported from India and sold in retail stores in Malaysia has recently been reported (32). However, STEC strains have not been able to cycle from contaminated food items or bovine reservoirs to humans. It is not certain whether the STEC strains in India lack some factor that is essential for them to become a frequent

cause of diarrhea or whether this phenomenon is related to an as-yet-unidentified host factor.

There was a disparity in the ability to culture STEC from PCR-positive specimens. STEC was cultured from 75% of PCR-positive human stools and 100% of community cows, but we isolated STEC from only 17.2% of PCR-positive cows from the livestock farm and 7.3% of PCR-positive raw beef samples. One reason for these varying results could relate to the initial numbers present in the sample. We presume that the number of strains present in human diarrhea cases (where the causative agent is amplified) would be higher than that found in fecal samples of cows and raw beef samples. However, we cannot account for the 100% isolation from community cows. We plan to investigate this further. One of the reasons for the failure to isolate STEC strains was the frequent presence of swarming colonies, which tended to obliterate all other colonies. This happened despite increasing the agar concentration (3%) as well as 0.15% bile salts (for the inhibition of swarming colonies) in the Luria agar medium. To avoid false-positive PCR results, we confirmed that the PCR amplicons were specific for *stx1* and *stx2* by dot blot assay. We immobilized the PCR product on N^+-nylon membrane and hybridized with *stx1*- and *stx2*-specific probes derived from pKTN501 and pKTN502, respectively, using the DIG-labeled kit. All PCR products gave positive signals in this hybridization assay, confirming that the PCR assay is specific for *stx1* and *stx2*.

The inability to isolate STEC strains from PCR-positive samples might be due to the presence of very low numbers of the target strain. This appears to be one key reason for the rather low isolation rate of STEC despite its being present in the sample. Several previous studies have also reported difficulties in isolating the organism from stools of patients with HUS and hemorrhagic colitis. In one such study of HUS cases in which 20 individual colonies of *E. coli* from primary stool cultures were tested, the proportion of Stx-positive colonies varied from 5% to 20% (33). Moreover, many cases had free fecal Stx but no STEC were isolated (34). In those studies, we usually encountered swarming colonies in the plates assayed for STEC even after the addition of 3% agar and bile salts (0.15%). In the present study, we used three different procedures to isolate the STEC and O157 strains. We usually had to screen approximately 500 colonies to yield a positive PCR result in an attempt to isolate STEC. But since the frequency of occurrence of STEC is very low and since PCR is an expensive and laborious method for screening, we later opted for the colony hybridization-DIG procedure, which allows a greater number of colonies to be initially screened and therefore increases the probability of obtaining positive isolates.

The systematic analysis of virulence markers indicates that the STEC in Calcutta mostly contain *stx1*, whereas the *eae* gene occurs at low frequency among strains of human and bovine origin. Such low prevalence of *eae* in Calcutta strains is in contrast to a report from Germany, where *eae* was found in most STEC strains examined (11). PCR results revealed that *hlyA* was the most prevalent (43.3%) plasmid-encoded marker

compared with *katP* (23.3%) and *etp*D (6.7%). With the *espP* gene, a considerable discrepancy was observed between the PCR and colony blot hybridization results. While 36.7% of strains were positive by colony blot hybridization, only 6.7% generated the desired amplicon with the *espP* primers. Comparison of the virulence profiles of Calcutta STEC strains isolated from different sources demonstrated the relative abundance of *katP* gene in cow and beef isolates (28.7% and 25%, respectively) compared with human strains (18.2%). Likewise, the percentage of *hlyA* PCR-positive STEC of bovine origin (64.3%) was more than twice that isolated from humans (27.3%). However, for the *espP* genes detected by colony hybridization, there was an appreciable variation in the distribution of this gene in strains isolated from cows (57.1%) and humans (18.2%). Two strains from cow stool samples were positive for *etpD*. Except for strains AK-40 and AK-38, nearly all Calcutta strains did not have the entire complement of virulence genes and therefore may not have been associated with diarrhea.

The observation that one *stx2*-positive strains showed cytotoxic effect on Vero cells but yielded negative Bead-ELISA result indicates that the toxin produced by this strain was antigenically different and may constitute a new variant of Stx2. Except for one strain, the other eight *hlyA*-positive STEC strains did not show the hemolytic phenotype when streaked on blood agar plates. One reason may be that the hemolysin of these strains remains cell associated because of a deficient transport system for secretion of hemolysin. Interestingly, one STEC strain AK-38 had all the known virulence markers of STEC but still gave a negative result in the Vero cell cytotoxicity assay. Based on the negative results obtained in Bead-ELISA and hemolysin assays, we presume that both *stx1* and *hlyA* genes of this strain (AK38) might be silent. Another possibility is that additional DNA is present in this isolate close to the *stx* gene, which would presumably prevent expression of *stx1* (11). Taken together, our analysis of non-O157 STEC strains suggests that pathogenicity is a consequence of lineal descent. The mere possession of *stx* genes is probably inadequate to render an *E. coli* pathogenic; an assortment of traits no doubt contributes to virulence, and the appropriate constellation of virulence traits appears to be present only in selected lineages.

In conclusion, this survey showed that STEC strains could not be implicated as a major causal agent of diarrhea but are present in the food chain in Calcutta. What exactly will trigger such strains to cause outbreaks is unclear. Given that STEC are present in the food chain in Calcutta and that STEC are not currently important human enteropathogens there, it would be useful to trace the natural history of the organism, should they become important enteropathogens in the not-too-distant future.

Mr. Asis Khan is a senior research fellow of the Indian Council of Medical Research. He is a doctoral student of Dr. G. Balakrish Nair, deputy director of the National Institute of Cholera and Enteric Diseases, Calcutta. Mr. Khan's research interests lie in the epidemiology of enteric pathogens.

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Address for correspondence: G. Balakrish Nair, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme XM, Beliaghata, Calcutta 700 010, India; fax: 91-33-350-5066; e-mail: gbnair@vsnl.com or gbnair@icddrb.org

Antimicrobial Drug Resistance in Pathogens Causing Nosocomial Infections at a University Hospital in Taiwan, 1981–1999

Po-Ren Hsueh, Mei-Ling Chen, Chun-Chuan Sun, Wen-Hwei Chen, Hui-Ju Pan, Li-Seh Yang, Shan-Chwen Chang, Shen-Wu Ho, Chin-Yu Lee, Wei-Chuan Hsieh, and Kwen-Tay Luh

To determine the distribution and antimicrobial drug resistance in bacterial pathogens causing nosocomial infections, surveillance data on nosocomial infections documented from 1981 to 1999 at National Taiwan University Hospital were analyzed. During this period, 35,580 bacterial pathogens causing nosocomial infections were identified. *Candida* species increased considerably, ranking first by 1999 in the incidence of pathogens causing all nosocomial infections, followed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Candida* species also increased in importance as bloodstream infection isolates, from 1.0% in 1981-1986 to 16.2% in 1999. The most frequent isolates from urinary tract infections were *Candida* species (23.6%), followed by *Escherichia coli* (18.6%) and *P. aeruginosa* (11.0%). *P. aeruginosa* remained the most frequent isolates for respiratory tract and surgical site infections in the past 13 years. A remarkable increase in incidence was found in methicillin-resistant *S. aureus* (from 4.3% in 1981-1986 to 58.9% in 1993-1998), cefotaxime-resistant *E. coli* (from 0% in 1981-1986 to 6.1% in 1993-1998), and cefotaxime-resistant *Klebsiella pneumoniae* (from 4.0% in 1981-1986 to 25.8% in 1993-1998). Etiologic shifts in nosocomial infections and an upsurge of antimicrobial resistance among these pathogens, particularly those isolated from intensive care units, are impressive and alarming.

The emergence of resistance to antimicrobial agents is a global public health problem, particularly in pathogens causing nosocomial infections (1-5). Antimicrobial resistance results in increased illness, deaths, and health-care costs (1,2,6-10). The distribution of pathogens causing nosocomial infections, especially antimicrobial-resistant pathogens, changes with time and varies among hospitals and among different locations in the same hospital (11-15). The increasing number of immunocompromised patients and increased use of indwelling devices, as well as widespread use of antimicrobial agents in hospital settings, particularly in intensive care units (ICUs), contributes to antimicrobial resistance among pathogens causing nosocomial infections (1,4,6,10).

Surveillance data reported by the National Nosocomial Infections Surveillance (NNIS) System for 1993-1997 compared with January-November 1998 show a continuing increase in antimicrobial-resistant pathogens associated with nosocomial infections in ICU patients from U.S. hospitals (2). The increase is particularly marked for vancomycin-resistant enterococci (VRE) (55%), methicillin-resistant *Staphylococcus aureus* (MRSA) (31%), third-generation cephalosporin-resistant *Escherichia coli* (29%), imipenem-resistant *Pseudomonas*

aeruginosa (32%), and quinolone-resistant *P. aeruginosa* (89%) (2). Studies since 1990 have clearly demonstrated that *Candida* species continue to be an important cause of nosocomial infections, particularly of bloodstream infections (BSI). Furthermore, the proportion of BSI caused by *Candida* species other than *C. albicans* is increasing (14,16-20).

We describe the distribution of major bacterial pathogens causing nosocomial infections from 1981 to 1999 in National Taiwan University Hospital and demonstrate the emergence of antimicrobial drug resistance among these pathogens during this period.

Materials and Methods

Data Collection

National Taiwan University Hospital is a 2,000-bed tertiary referral center in Taipei, northern Taiwan. Available data for inpatient-days at the hospital ranged from 294,946 in 1990 to 566,165 in 1999. The number of ICU beds increased from 40-50 before 1993 to 100-120 in 1998-99. The Nosocomial Infection Control Committee of the hospital was established in 1980 to identify pathogens causing nosocomial infections and to obtain and analyze antimicrobial susceptibility results of these pathogens from the hospital's clinical microbiology laboratory. NNIS definitions were used for nosocomial infections (e.g.,

National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan

bloodstream; respiratory tract, including lower respiratory tract and pneumonia; urinary tract; and surgical site infections) (21,22). Isolates were considered nosocomial if the culture was dated >2 days after admission. All isolates were identified by standard methods and confirmed by using Vitek or API products (bioMerieux Vitek, Inc., Hazelwood, MO). For determining the percentage of resistance, the same organisms from multiple blood cultures or from the same sources with identical antibiotype were considered a single isolate. The amount of use for each indicated antimicrobial agent (including oral and parenteral forms) was expressed in grams per 1,000 inpatient-days.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of the bacterial isolates was performed by the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (23). *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were included as control strains. Interpretive criteria for susceptibility or resistance followed NCCLS guidelines (23). For this report, we present susceptibility data for penicillin, oxacillin, vancomycin, gentamicin, cefotaxime, ceftazidime, imipenem, and ciprofloxacin. The susceptibility data for imipenem and ciprofloxacin were available only since 1993; those for other agents were available from 1981 to 1999.

Antifungal susceptibility testing of amphotericin B and fluconazole against 150 blood isolates of *Candida* species collected from October 1997 to September 1999 was performed by the E test (AB BIODISK, Solna, Sweden) according to the manufacturer's instructions. Quality control was performed by testing *C. parapsilosis* ATCC 2019 and *C. krusei* ATCC 6258. MIC results were interpreted in accordance with NCCLS guidelines (24).

Results

During the 19-year period, 35,580 bacterial pathogens causing nosocomial infections were identified. The hospital's overall rate of nosocomial infections during the 19-year period ranged from 3.9% to 6.1%. For the four major sites of nosocomial infections, data are presented as numbers of infection per 10,000 patient-days from 1991 to 1999 (Figure 1). BSI ranked first in nosocomial infection sites in 1999, followed by urinary tract, surgical site, and respiratory tract infections.

Data for the catheter- and ventilator-days of the hospitalized patients were not available. However, the mean percentages of patients who stayed in ICUs (six medical ICUs and six surgical ICUs) and used urinary catheters, arterial and central venous catheters, and ventilators were 75.0%, 77.9%, 63.2%, respectively, in 1996 and 79.4%, 81.4%, and 65.2%, respectively, in 1999. The incidence (number of infections/patient-days x 1,000) of urinary catheter-related urinary tract infection, vascular catheter-associated BSI, and ventilator-associated pneumonia in the ICUs was 4.4, 5.6, and 2.7, respectively, in 1996 and 6.0, 7.1, and 3.4, respectively, in 1999.
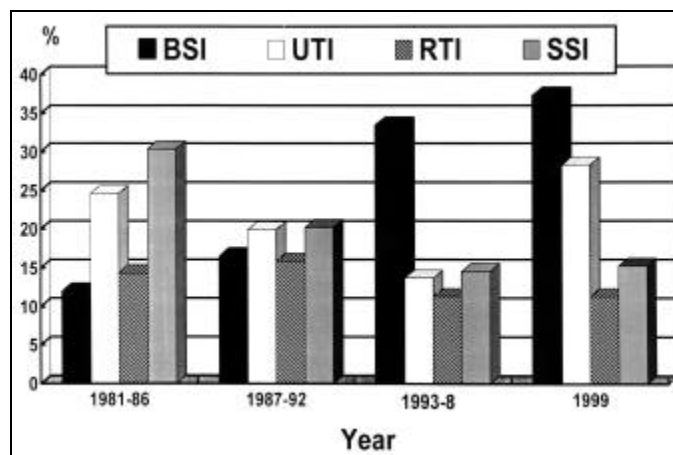

Figure 1. Rates of four major nosocomial infections expressed as number of infections per 10,000 patient-days at National Taiwan University Hospital from 1991 to 1999. BSI, bloodstream infection; UTI, urinary tract infection; SSI, surgical site infection; RTI, respiratory tract infection.

Gram-negative bacteria remained the predominant pathogens (66.1% in 1981, 51.3% in 1993, and 53.4% in 1999); however, incidences of fungal infections have increased recently (Table 1). In a comparison of data grouped into four time periods (1981-1986, 1987-1992, 1993-1998, and 1999), *E. coli* decreased from 12.1% of all nosocomial infections in 1981-1986 to 9.5% in 1993-1998; however, infections caused by *S. aureus* increased from 5.2% (1981-1986) to 12.1% (1993-1998). *Candida* species showed a considerable increase (from 3.7% in 1981-1986 to 16.2% in 1999) and have ranked first since 1993, followed by *S. aureus* and *P. aeruginosa* (Table 1).

Candida species, *S. aureus,* and *Acinetobacter* species were also important bloodstream isolates (Table 1), increasing from 1.0%, 5.2%, and 6.1%, respectively, in 1981-1986 to 16.2%, 13.0%, and 7.6%, respectively, in 1999. Although *E. coli* was also a frequent isolate, it declined in percentage of all BSI from 18.7% in 1981-1986 to 9.0% in 1999. Other pathogens declining in percentage of BSI from 1981-1986 to 1999 included *K. pneumoniae, P. aeruginosa*, and *Enterococcus* species.

From 1992 to 1999, 1,065 isolates of *Candida* species were recovered from patients with nosocomial BSIs. *C. glabrata* (8.8%) ranked fourth in incidence behind *C. albicans* (59.3%), *C. tropicalis* (17.6%), and *C. parapsilosis* (8.2%) in 1999. Only four blood isolates of *C. krusei* were identified during the 8-year period.

The most frequent isolates from urinary tract infections in 1993-1998 were *Candida* species (23.6%), followed by *E. coli* (18.6%) and *P. aeruginosa* (11.0%). However, in 1999, *E. coli* (18.4%) replaced *Candida* species (14.3%) as the top-ranking pathogen causing urinary tract infections. *P. aeruginosa* remained the most frequent isolate for respiratory tract and surgical site infections in the past 13 years. *Candida* species increased in incidence in surgical site infections, from 1.8% in 1981-1986 to 6.4% in 1999. Among pathogens causing respiratory tract infections, *Acinetobacter* species ranked fifth in

Table 1. Incidences of 10 top-ranking pathogens causing nosocomial infections and infections from four body sites at National Taiwan University Hospital, 1981 to 1999

Pathogen	% of indicated pathogen causing nosocomial infection (1981-1986/1987-1992/1993-1998/1999)[a]				
	All	BSI	RTI	UTI	SSI
Candida spp.	3.7/9.1/14.4/16.2	1.0/9.2/16.4/16.2	2.0/5.8/2.1/2.2	8.4/16.0/23.6/14.3	2.4/5.1/5.9/6.4
Staphylococcus aureus	5.2/9.1/12.1/12.0	5.2/9.3/11.5/13.0	4.0/8.4/16.9/12.6	1.4/2.6/3.3/2.1	5.5/5.2/13.0/15.4
Pseudomonas aeruginosa	12.7/14.0/11.1/11.8	10.0/9.4/7.2/7.8	19.6/21.9/23.8/25.7	11.7/11.2/11.0/10.4	11.1/17.4/14.3/16.0
Escherichia coli	12.1/8.4/9.5/9.9	18.7/9.7/8.7/9.0	4.8/2.4/3.5/3.7	19.1/19.9/18.6/18.4	11.7/5.8/5.8/6.8
Klebsiella pneumoniae	8.1/5.5/7.2/6.8	11.6/6.6/7.7/7.0	10.9/9.4/11.5/10.8	9.0/7.0/8.6/8.2	6.9/3.5/4.2/4.6
Enterobacter spp.	6.0/7.6/7.4/6.4	8.0/8.6/7.3/6.9	5.2/8.1/11.8/8.6	9.0/8.4/6.7/6.3	4.5/7.9/7.8/5.5
Enterococcus spp.	8.8/7.8/6.7/6.2	8.7/6.2/6.3/7.6		11.6/9.7/8.1/6.5	10.1/12.6/9.6/7.9
Acinetobacter spp.	4.4/5.1/4.9/5.4	6.1/8.8/7.2/7.6	11.0/13.4/9.3/13.0		
CoNS	2.8/6.9/6.6/5.1	2.7/8.5/7.9/4.9			3.2/7.1/9.1/6.8
Other NFGNB	5.7/6.1/4.8/4.1	5.9/7.7/6.8/6.7	12.3/13.1/7.8/8.2	7.4/6.5/4.4/2.8	
S. marcescens			3.5/1.9/3.7/4.1		
Proteus spp.			3.2/2.2/2.4/1.1	3.8/3.8/3.8/3.7	
Citrobacter spp.				5.9/4.4/2.4/2.4	
Viribans streptococci					5.2/5.8/4.2/3.5
Bacteroides spp.					9.7/4.7/5.0/3.1

[a]Abbreviations: BSI = bloodstream infection; RTI = respiratory tract infection; UTI = urinary tract infection; SSI = surgical site infection; CoNS = coagulase-negative staphylococci; NFGNB = nonfermentative gram-negative bacilli.

1993-1998 (9.3%) but second in 1999 (13.0%); *Candida* species accounted for only 2.2% in 1999.

The distributions of selected antimicrobial drug-resistant pathogens causing all nosocomial infections and BSIs of patients hospitalized in intensive care units or general wards is shown in Table 2. Antimicrobial drug-resistant pathogens causing BSIs that increased markedly over the study period were methicillin-resistant *S. aureus* (4.3% in 1981-1986, 58.9% in 1993-1998, and 69.2% in 1999), cefotaxime-resistant *E. coli* (0% in 1981-1986, 6.1% in 1993-1998, and 12.5% in 1999), and cefotaxime-resistant *Klebsiella pneumoniae* (4.0% in 1981-1986, and 25.8% in 1993-1998). The frequencies of these three resistant pathogens were considerably higher in isolates from ICUs than those from general wards (84.6% vs. 48.3% for MRSA, 17.1% vs. 5.1% for cefotaxime-resistant *E. coli*, and 51.1% vs. 18.3% for cefotaxime-resistant *K. pneumoniae* in 1993-1998). The incidence of methicillin-resistant coagulase-negative staphylococci (MRCoNS) remained high (72%-90%) during the 19-year period.

Ceftazidime use is associated with trends of several antimicrobial-resistant pathogens during the period 1991 to 1999 (Figure 2). Restriction of third-generation cephalosporins (particularly ceftazidime) was implemented in 1997. In 1999, resistance to cefotaxime in *K. pneumoniae* diminished; however, resistance to cefotaxime in *E. coli* and resistance to ceftazidime in *P. aeruginosa* slightly increased.

The first clinical isolate of VRE was recognized in 1995 (25,26). Since then, 80 isolates of VRE (49 of *E. faecalis* and 31 of *E. faecium*) have been recovered from hospitalized patients. The incidence of VRE in isolates causing nosocomial infection increased from 1.8% in 1995 to 6.7% in 1997 and 25.2% in 1999 (Figure 3). Among these VRE isolates, 4 were from blood, 15 from urine, and the rest were pus or drainage fluid. Only 12 (15%) of these isolates were from patients admitted to ICUs, and 6 of these 12 isolates were recovered in 1999. The incidence of VRE in enterococci causing nosocomial infections in ICUs was 7.0%. The relationship of increasing vancomycin use and the increase in vancomycin resistance in enterococci is shown in Figure 3.

The incidences of imipenem-resistant *P. aeruginosa* (1993 to 1998) and imipenem-resistant *Acinetobacter baumannii* (1999) isolated from ICUs were five- to ten-fold higher than isolates recovered from non-ICU settings. However, this was not the case with imipenem-resistant *P. aeruginosa* in 1999 or imipenem-resistant *A. baumannii* in 1993-1998 (Figure 4).

All 150 isolates of *Candida* species were inhibited by 1 µg/mL of amphotericin B (MIC range 0.03 to 1 µg/mL). The MIC_{50} and MIC_{90} of *C. glabrata* were 16 µg/mL and >32 µg/mL, respectively. Twenty-one isolates (70%) of *C. glabrata* were nonsusceptible to fluconazole (MICs >8 µg/mL). Four (15.4%) of the *C. parapsilosis* isolates had MICs >8 µg/mL (2 had MICs 16 µg/mL and the other 2 had MICs 64 µg/mL).

Table 2. Selected antimicrobial resistant pathogens associated with nosocomial infection at National Taiwan University Hospital from 1981 to 1999

Resistant pathogen	% resistance (all patients/in intensive care unit patients/in patients in general wards)			
	1981-1986	1987-1992	1993-1998	1999
Methicillin-resistant *Staphylococcus aureus*	20.2/27.8/19.5	31.4/58.5/26.6	64.8/86.9/56.7	69.3/87.4/60.2
Methicillin-resistant CoNS[a]	72.2/88.2/70.8	74.0/83.9/71.5	79.0/88.7/75.1	90.6/90.9/90.5
Penicillin-resistant enterococci	5.9/6.8/5.8	20.7/30/19.6	19.5/22.6/18.7	35.9/40.9/27.8
Gentamicin-resistant enterococci	0.0/0.0/0.0	71.0/71.4/70.9	61.5/67.2/60.0	50.0/39.3/55.2
Cefotaxime-resistant *Escherichia coli*	1.6/0.0/1.7	2.8/8.6/2.5	6.8/13.1/6.2	12.3/10.3/12.7
Cefotaxime-resistant *Klebsiella pneumoniae*	4.9/8.8/3.9	7.4/16.9/5.6	22.8/50.5/15.6	16.5/40.0/9.3
Cefotaxime-resistant *Enterobacter* spp.	35.8/52.4/32.4	49.7/55.7/47.7	57.6/67.0/53.6	50.9/61.8/46.2
Cefotaxime-resistant *Pseudomonas aeruginosa*	24.2/20.0/25.0	16.1/24.3/12.1	10.2/16.9/7.8	11.2/17.6/8.1
Imipenem-resistant *P. aeruginosa*	NA	NA	8.5/18.2/4.9	6.7/4.5/7.7
Ciprofloxacin-resistant *P. aeruginosa*	NA	NA	8.7/11.6/5.8	14.0/18.2/2.9
Imipenem-resistant *Acinetobacter baumannii*	NA	NA	6.7/9.1/4.4	12.5/23.3/7.6

[a]CoNS = coagulase-negative staphylococci; NA = not available.

Discussion

Hospitals worldwide are continuing to face the crisis of the upsurge and dissemination of antimicrobial-resistant bacteria, particularly those causing nosocomial infections in ICU patients (1,27-29). Among resistant bacteria, MRSA, MRCoNS, VRE, third-generation cephalosporin-resistant *Enterobacteriaceae*, and imipenem- or ciprofloxacin-resistant *P. aeruginosa* and *A. baumannii* are of great concern because these bacteria have spread worldwide and ultimately will compromise the antimicrobial therapy of infections caused by these organisms (2,25-28,30).

This report describes trends in major nosocomial pathogens and shifts in antimicrobial resistance during a 19-year period in a large teaching hospital in Taiwan. In a comparison of data from a recent NNIS study and other surveillance systems (2,4,9,31), our results suggest four conclusions. First, *Candida* species, rather than *P. aeruginosa*, *E. coli*, or staphylococci, now are the most frequent pathogens causing overall nosocomial infections and BSIs in this hospital. The upward trend in coagulase-negative staphylococci, which was the leading cause of BSI in the recent NNIS study, was not confirmed in our study. Second, we observed a remarkably high incidence of MRSA, especially in ICUs, contrasted with a relatively low incidence of VRE. Third, we report an increase in incidence of cefotaxime-resistant *Enterobacteriaceae*, especially in the incidence of cefotaxime-resistant *K. pneumoniae* in ICUs. Fourth, although the overall incidence of imipenem resistance among *P. aeruginosa* and *A. baumannii* in recent years has remained
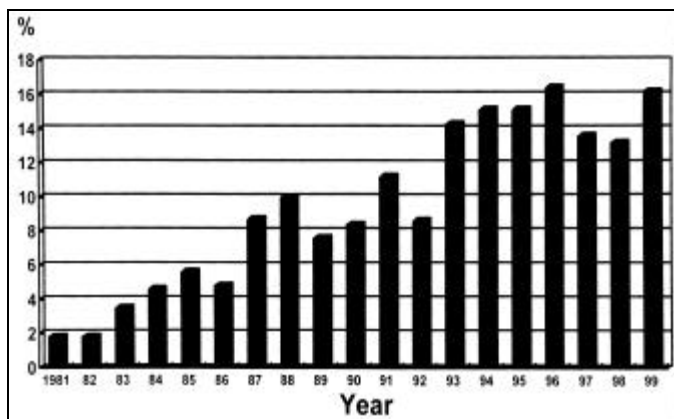
Figure 2. Changes in major antimicrobial-resistant nosocomial pathogens in relation to ceftazidime use at National Taiwan University Hospital from 1991 to 1999.
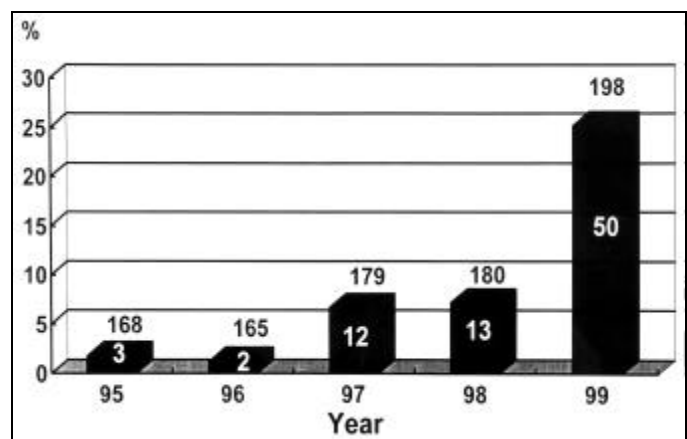
Figure 3. Incidences of vancomycin-resistant enterococci (VRE) among all enterococcal isolates causing nosocomial infections in relation to vancomycin use at National Taiwan University Hospital, 1995-1999. Numbers above the bars denote the number of enterococcal isolates causing nosocomial infections. Numbers within the bars denote the numbers of VRE.
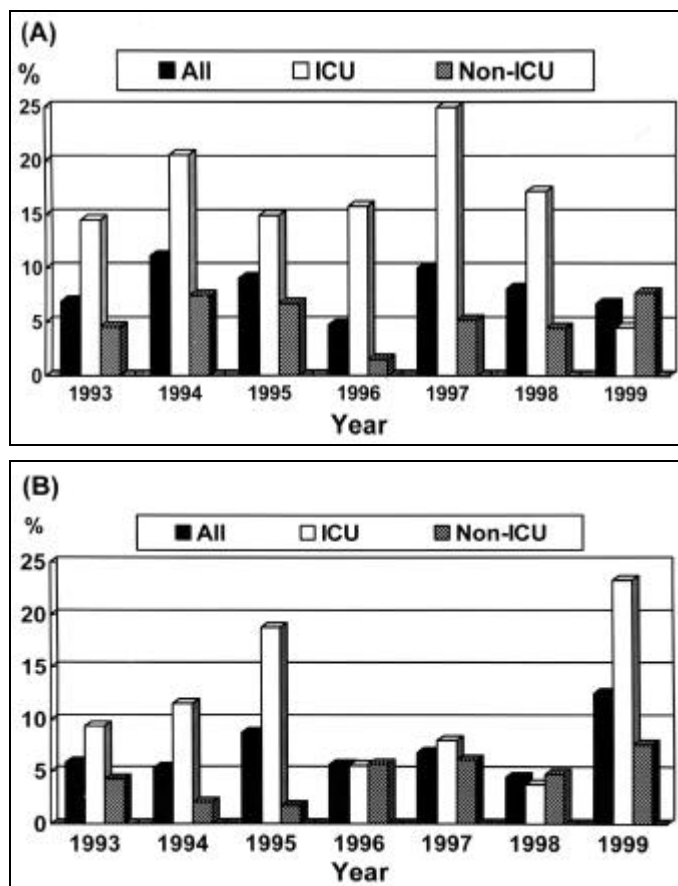
Figure 4. Proportions of *Pseudomonas aeruginosa* (A) and *Acineto-bacter baumannii* (B) isolates causing nosocomial infections resistant to imipenem in National Taiwan University Hospital, 1993-1999. ICU = intensive care unit.

stable (4% to 11%), higher incidences of imipenem-resistant *P. aeruginosa* or *A. baumannii* were found in ICUs than in general wards in most time periods.

Since 1990, *Candida* species have continued to be an important cause of nosocomial BSI in the United States, and the proportion (40%-50%) of these infections due to species of *Candida* other than *C. albicans* may be increasing (12,19,20,31,32). Among the species of *Candida* other than *C. albicans, C. glabrata* (prone to be resistant to fluconazole) and *C. krusei* (intrinsically resistant to fluconazole) are of clinical importance (31,33). Although the proportion (40%) of candidemia due to non-*albicans Candida* species in 1999 in our hospital was similar to that (48%) reported in the United States from April 1995 to June 1996, the incidences of *C. glabrata* (8.8%) and *C. krusei* (0%) in our hospital were lower than those (20% and 5%, respectively) in the United States (31).

Although an upsurge in the incidences of *K. pneumoniae* and *E. coli* isolates resistant to cefotaxime was noted in our ICUs, an investigation is under way into the mechanisms of resistance and potential outbreaks (clonal dissemination or gene transfer) (34). The abrupt increase in the proportion of *A. baumannii* isolates resistant to imipenem in 1999 resulted from wide dissemination of several multidrug-resistant clones in ICUs and many general wards in the hospital (data not shown).

In summary, surveillance of the microbial etiology of nososcomial infections over prolonged time periods not only can provide important information for day-to-day decision making in antimicrobial therapy in individual hospitals but also can reflect local trends and shifts in etiology and antimicrobial drug resistance. Nosocomial pathogens have shifted away from easily treated bacteria toward more resistant bacteria and even to *Candida* species with fewer options for therapy. These shifts continue to present challenges for nosocomial infection control and prevention.

Dr. Hsueh is an assistant professor in the departments of Laboratory Medicine and Internal Medicine, National Taiwan University College of Medicine. His research interests include epidemiology of emerging and nosocomial infections and mechanisms of antimicrobial drug resistance. He is actively involved in developing a national research program for antimicrobial drug resistance (Surveillance for Multicenter Antimicrobial Resistance in Taiwan).

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Address for correspondence: Kwen-Tay Luh, Department of Laboratory Medicine, National Taiwan University Hospital, No. 7 Chung-Shan Road, Taipei 100, Taiwan; fax: 886-2-23224263; e-mail: luhkt@ha.mc.ntu.edu.tw

Tularemia Outbreak Investigation in Kosovo: Case Control and Environmental Studies

Ralf Reintjes,* Isuf Dedushaj,† Ardiana Gjini,‡ Tine Rikke Jorgensen,§ Benvon Cotter,¶#
Alfons Lieftucht,#** Fortunato D'Ancona,¶ David T. Dennis,†† Michael A. Kosoy, Gjyle
Mulliqi-Osmani,† Roland Grunow,‡‡ Ariana Kalaveshi,† Luljeta Gashi,† and Isme Humolli†

A large outbreak of tularemia occurred in Kosovo in the early postwar period, 1999-2000. Epidemiologic and environmental investigations were conducted to identify sources of infection, modes of transmission, and household risk factors. Case and control status was verified by enzyme-linked immunosorbent assay, Western blot, and microagglutination assay. A total of 327 serologically confirmed cases of tularemia pharyngitis and cervical lymphadenitis were identified in 21 of 29 Kosovo municipalities. Matched analysis of 46 case households and 76 control households suggested that infection was transmitted through contaminated food or water and that the source of infection was rodents. Environmental circumstances in war-torn Kosovo led to epizootic rodent tularemia and its spread to resettled rural populations living under circumstances of substandard housing, hygiene, and sanitation.

K osovo (pop. approximately 2 million) has undergone severe social and economic disruption. By mid-1999, more than 10 years of political crisis and warfare had resulted in environmental disruption, mass population displacements, and a breakdown of sanitation and hygiene. Many essential public health functions, such as disease surveillance and outbreak response, had collapsed. In January 2000, the Kosovo Institute of Public Health (IPH) in Pristina implemented a new surveillance system for 20 communicable disease syndromes. On March 22, 2000, a public health physician in western Kosovo reported a cluster of patients with an unusual syndrome of fever, pharyngitis, and pronounced cervical lymphadenitis. Tularemia was clinically suspected, and the diagnosis was serologically confirmed on April 14 at the World Health Organization (WHO) regional reference laboratory in Rome (1). Active case-finding identified other patients at multiple sites in Kosovo who in the previous 6 months had a similar syndrome. Public health records as far back as 1946 disclosed no prior reports of tularemia in Kosovo, but the disease has been reported, although infrequently, from other areas of Yugoslavia and other Balkan states (2).

Tularemia, a zoonotic disease caused by the highly infective, virulent, nonsporulating gram-negative coccobacillus

Francisella tularensis, is found throughout most of the Northern Hemisphere in a wide range of animal reservoir hosts. In addition, the organism can be isolated from contaminated environmental sources such as water and mud. It is transmitted to humans by various modes, including direct handling of infectious carcasses, ingestion of contaminated food or water, arthropod bites, or inhalation of infectious dusts or aerosols. Person-to-person transmission is not known to occur. Epidemics can often be traced to concurrent epizootics involving rodents and lagomorphs (rabbits and hares) (3,4), which may be associated with unusual increases in population density of these animal hosts. Generalized symptoms of tularemia typically have sudden onset, often with high fever, chills, fatigue, headache, pharyngitis, sore joints, chest discomfort, dry cough, vomiting, abdominal pain, and diarrhea. The usual incubation period is 3 to 5 days, although it can be as long as 21 days.

In humans, there are several tularemia syndromes, mostly depending on the portal of infection. Ingestion typically results in oropharyngeal tularemia, with fever, pharyngitis, cervical lymphadenitis, and suppuration (Figure 1). Other tularemia syndromes include ulceroglandular, glandular, pleuropneumonic, and systemic (typhoidal) forms (5). Severity of illness is related to biotype: *F. tularensis* biovar palaearctica (Type B), which causes tularemia in Europe, is less virulent than *F. tularensis* biovar tularensis (Type A), which is associated with sometimes severe and fatal illness in North America (6). Without antimicrobial treatment, tularemia can be acute and fulminant or protracted and debilitating.

Suspecting a widespread outbreak of tularemia in Kosovo, on April 14, 2000, the United Nations Mission in Kosovo requested WHO to assist Kosovar health authorities in an epidemiologic investigation. Teams of international and Kosovar

*Institute of Public Health North Rhine-Westphalia, Muenster, Germany; †Institute of Public Health, Pristina, Kosovo; ‡World Health Organization, Pristina, Kosovo; §World Health Organization Regional Office for Europe, Copenhagen, Denmark; ¶ Istituto Superiore di Sanita, Rome, Italy; #European Programme for Intervention Epidemiology Training, Paris, France; **PHLS Communicable Disease Surveillance Centre, London, United Kingdom; ††Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; and ‡‡German Reference Laboratory on Tularemia, Munich, Germany

Figure 1. Girl with ulcerating lymphadenitis colli due to tularemia, Kosovo, April 2000.

public health personnel collaborated in field and laboratory investigations of epidemiologic, environmental, and microbiologic factors. The aims were to identify infection sources, vehicles, and modes of transmission and recommend appropriate control measures. In this report, we describe a case-control study of household risk factors and supporting environmental observations.

Methods

Surveillance

In early April 2000, local authorities initiated active tularemia case finding by enhanced retrospective and prospective surveillance. Hospitals and clinics in Kosovo were asked to report all cases of illness with fever, lymphadenitis, and skin ulceration since the summer of 1999. Particular attention was paid to a syndrome of pharyngitis and cervical lymphadenitis. Investigators conducted in-depth interviews of persons with suspected cases during visits to homes. In addition, these ill persons were clinically examined and interviewed; if the illness was presumed to be tularemia, blood samples were drawn for serology and patients were treated with antibiotics, as appropriate. During the interviews, rural villagers frequently reported that the numbers of mice and rats had exploded in the spring and summer of 1999. A causal association between increased population density of rodents and human tularemia was hypothesized, based on suspected contamination of the environment by *F. tularensis*-infected rodents.

Case-Control Study

To test the hypothesis that a rodent-contaminated environment was the source of human tularemia and to determine household risk factors for infection, a matched case-control study was conducted with paired households as study units.

Case households were defined as households with one or more family members having a laboratory-confirmed case of tularemia since November 1, 1999. Control households were defined as the two households closest to a case household, with no family member having a history of a syndrome of fever, pharyngitis, and cervical lymphadenitis since November 1, 1999, and with the person who prepared the family's food being serologically negative for tularemia. Blood specimens were drawn from all persons with suspected cases, and in the control households, blood was drawn from the person responsible for preparing the household food. Structured questionnaires were completed on household food consumption, water supply, and presence of rodents in fields and domestic environments, and observations were recorded on conditions of wells and food preparation and storage areas. The study period began a month before onset of symptoms of the first case in the case household. The case and control household investigations were conducted in small villages in rural farming areas in Peje, Istog, Kline, and Deqan municipalities in the Peje Region, and in several villages in the adjacent Gjakova Region, western Kosovo (Figure 2). These regions were selected for study because they had the greatest numbers of reported cases.

Bivariate statistical analyses were performed in a matched pair analysis by chi-square test and Fisher's exact test. Odds ratios (OR) (95% confidence intervals [CI]) were calculated by Epi Info 6.04 (7). Multivariate analyses were performed by conditional logistical regression by using a backward elimination model with Logistics (8).

Environmental Investigations

Rodents were collected by baited live traps placed in homes, outbuildings, gardens, and fields of selected case households and neighboring households. Captured rodents were euthanized and identified to species, and blood, liver, and spleen specimens were collected. Blood samples were kept refrigerated and spun within 24 hours for separation of serum.

Figure 2. Total number of confirmed tularemia cases in Kosovo by municipality, July 1999-May 2000.

Homes, outbuildings, dug wells, and other water sources were examined for evidence of contamination by rodent carcasses or excrement. Several rodent carcasses were collected from wells. Fecal specimens were collected from captured animals and from food storage and preparation areas of case and neighboring households. Water samples were collected from some wells and streams, and mud samples were collected from well heads and streams.

Serologic testing was done for immunoglobulin (Ig) M, IgG, and IgA isotype-specific antibodies by enzyme-linked immunosorbent assay (ELISA), microagglutination assay for screening, and Western blot for confirmation (9). Rodent tissues and feces were tested for *F. tularensis* lipopolysaccharide (LPS) by antigen-capture ELISA (10). Growth of *F. tularensis* on culture media was not attempted.

Results

Descriptive Epidemiology

By June 30, 2000, >900 suspected cases of tularemia had been identified by IPH. This total included cases identified by retrospective record review, as well as prospectively by hospitals and clinics and by health teams conducting village surveys. Of these ill cases, 912 had serologic examinations and 327 were confirmed as tularemia positive. The earliest onset of reported symptoms in any of the confirmed cases occurred in October 1999. The epidemic curve of confirmed cases shows a peak in January 2000 (Figure 3). Confirmed cases were identified in 21 of 29 municipalities in Kosovo. (Several municipalities under Serbian authority did not submit data to IPH Kosovo; however, no cases from these areas were reported to IPH Belgrade [unpub. data, WHO, Belgrade]). Most cases were found in the western part of Kosovo (Figure 2), almost all from rural areas. Cases were equally distributed by sex (female 51.8%; male 48.2%), and all age groups were affected (median age 18 years; range <1 to 76 years).

Analytical Epidemiology

To test the principal hypothesis that tularemia was associated with the consumption of food or water contaminated by *F. tularensis*-infected rodents, 46 case households and 76 control households were included in a matched analysis. Visual inspection of the case and control households confirmed the similarity of environmental and socioeconomic living conditions. Bivariate matched pair analyses of questionnaire data indicated that members of case households were less likely to have eaten fresh vegetables than were control households (Table 1). Case households were more likely to have water sources (mostly crude, open wells) unprotected against rodents (Table 1). Conditional logistic regression modeling indicated these independent risk factors for a case household: rodent feces in food preparation and storage areas (OR 5.7; 95% CI 1.5-22.2 [Table 2]) and large numbers of field mice seen outside the house (OR 5.6; 95% CI 1.1-28.7). The greater odds ratio for observing field mice obtained by multivariate analysis compared with
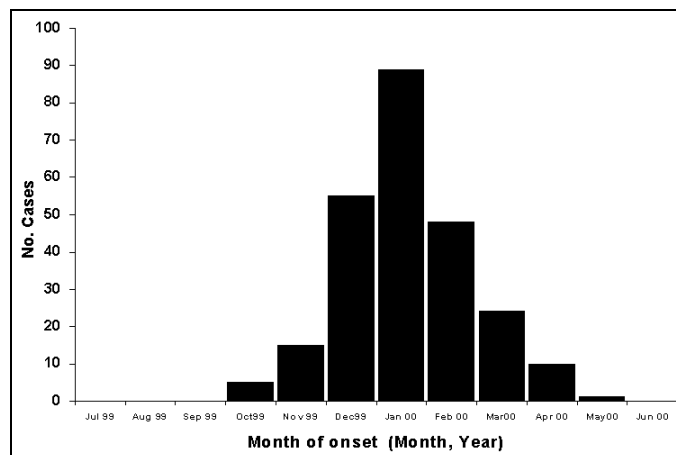

Figure 3. Epidemic curve of laboratory-confirmed tularemia cases (n = 247) in Kosovo, by month of onset of symptoms, October 1999–May 2000.

bivariate analysis suggests either interaction of terms in the regression model or confounding in the bivariate analysis, although we were unable to identify either. Two factors were independently protective: household water source protected against rodents (OR 0.2; 95% CI 0.02-0.99) and eating fresh vegetables (OR 0.1; 95% CI 0.01-0.8) (Table 2).

Environmental Findings

Villages in the affected regions are mostly located in a broad valley with rolling hills, surrounded by steep foothills and mountains in an arc from northwestern to southwestern Kosovo. The Peje Valley has a confluence of rivers from surrounding elevations and is intersected by streams. The environment appeared favorable for the common epizootic hosts of *F. tularensis* in Europe (water voles [*Arvicola terrestris*] and common voles [*Microtus arvalis*]), but villagers did not describe these rodents, and investigators saw no signs of them. Sites of large colonies of voles were, however, identified in subalpine meadows in the mountains west of the valley, but no trapping was done there. Residents of affected villages readily identified the striped field mouse and the black rat as the animals that had overrun their properties in 1999. In addition, in late summer 1999, villagers noticed unusually large numbers of weasels, which are important rodent predators.

Table 1. Household risk factors for tularemia determined by bivariate matched comparison of 46 case households and 76 control households, Kosovo, October 1999-May 2000

Risk factor	OR[a]	95% CI	p-value
Rodent feces in food storage	3.6	1.1-9.8	0.01
Large numbers of field mice near house	2.6	0.6-6.3	0.2
Piped water as water source	2.0	0.1-176.8	0.4
Personal well as water source	1.4	0.3-4.0	0.5
Well protected from rodents	0.3	0.1-1.1	0.04
Eating fresh vegetables	0.2	0.02-0.9	0.02

[a]OR = odds ratio; CI = confidence interval.

Table 2. Risk factors for tularemia determined by conditional logistic regression in a matched comparison of 46 case households and 76 control households, Kosovo, October 1999–May 2000

Variable	OR[a]	95% CI	p value
Rodent feces in food storage	5.8	1.5-22.2	0.01
Large numbers of field mice near house	5.7	1.1-28.7	0.04
Well protected from rodents	0.2	0.02-0.99	0.04
Eating fresh vegetables	0.1	0.01-0.80	0.03

[a]OR = odds ratio; CI = confidence interval.

Homes were constructed of cement, stones or bricks, and tile roofs supported by wooden frames; almost all had been severely damaged by fire and explosives and were in various stages of repair. Most were surrounded by yards that included common work areas; small gardens; wells of primitive stone construction with absent or inadequate cement aprons, head cylinders, or protective covers; and outdoor latrines without pits. Rodents had easy access to most wells, where they could find harborage in the rock walls. Livestock and domestic pets were sheltered in crude outbuildings and sometimes on the ground level of residences. Feed corn was stored in cribs with easy rodent access; other animal foodstuffs were typically stored unprotected in open rooms in homes and outbuildings. Fields and gardens were near homes, many were fallow or newly cultivated, and some contained unharvested crops. Household food storage and preparation areas were often poorly protected from rodents and were frequently contaminated by rodent feces.

Sixty-four rodents representing five species were collected: 26 striped field mice (*A. agrarius*); 2 yellow-necked mice (*A. flavicollis*); 2 wood mice (*A. sylvaticus*); 23 house mice (*Mus musculus*); and 11 black rats (*Rattus rattus*). Tissues from these species were tested at the temporary WHO tularemia investigative laboratory in Pristina. *F. tularensis* LPS antigen was detected by antigen-capture ELISA in the liver tissue of *A. agrarius* (Deqan municipality) and in an *A. agrarius* recovered from a well in the village in Gjakova, where the index cases were reported. Of 48 mammalian fecal specimens collected and tested for *F. tularensis* antigen, 5 were antigen positive, 3 from *A. agrarius* and 2 from *R. rattus*.

Discussion

The clinical picture of fever, pharyngitis, and cervical lymphadenitis, together with serologic confirmation of several hundred cases, indicates that an outbreak of tularemia occurred throughout rural Kosovo during October 1999 to May 2000. The results of the case-control study support the hypothesis that tularemia was foodborne, based on associations of illness with large numbers of rodents in the peridomestic environment, rodent contamination of food storage and preparation areas, and eating some uncooked foods. In addition, epidemiologic and environmental evidence suggested that unprotected and unboiled water contributed to the outbreak. We were not able to explain a possible independent protective value of eating fresh (unprocessed) vegetables, but it may be related to shorter storage and less chance of contamination, a surrogate for better socioeconomic circumstances or less disruption of farming, or an association attributable to chance alone.

This is the first outbreak of tularemia described in Kosovo. The unexpected finding of tularemia immediately after warfare raised questions about the origin of the outbreak and its epidemiologic characteristics. Some authorities expressed concern about possible intentional use of *F. tularensis*, since this pathogen is considered a class A agent for possible bioterrorism use (11). However, initial field investigations quickly provided evidence of a widely occurring natural event, most likely resulting from unusual environmental and sociopolitical circumstances in war-torn Kosovo.

The most recent Kosovo census was done in 1981, when reliable population estimates were not available. Since there were no laboratories in Kosovo equipped to work with tularemia, an emergency laboratory was set up to deal with the outbreak samples. Under these circumstances, we performed a matched case-control study of households supported by limited environmental investigations during April to May 2000.

Results of the case-control study were strongly supported by the environmental investigations. The principal populations affected by the tularemia outbreak were ethnic Albanians with limited economic means, living in rural farming villages. These populations had precipitously fled to neighboring areas in the mass exodus during NATO bombing and Serbian reprisals in spring 1999. When refugees returned weeks to months later, they found a vastly disordered environment. Most homes had been ransacked and destroyed by incendiary devices and explosives, food storage areas had been left unprotected, wells had been damaged and contaminated, crops had been left unharvested, and many fields were fallow. Most striking, the returning refugees noted a population explosion of rodents in fields, gardens, homes, and outbuildings. The increased rodent activity in and around homes was reported to have peaked in fall 1999 and continued to be a problem in homes during the winter months. By the time of our investigation, rodent levels had returned to normal. The investigative teams readily found signs of rodent activity in houses and rodent contamination of wells and food materials.

Some rodent tissues and feces sampled contained *F. tularensis* LPS antigen. Although it was considered important to isolate *F. tularensis* from clinical and environmental samples, facilities were not available for growing the organism in culture in Kosovo, and authorities were concerned with the hazards this could pose to laboratory workers.

A disrupted agricultural environment, deserted homes, and unprotected food stores in Kosovo in spring 1999 likely resulted in a rapid increase in rodent populations favorable for epizootic spread of tularemia in rodents and consequent widespread environmental contamination with *F. tularensis*. Although this organism does not produce spores or multiply outside animal hosts, it can survive for months in cold, moist

conditions. Large outbreaks of human tularemia in Europe have been described following contamination of the environment with rodent excrement and carcasses (12-18). The largest occurred as a result of disrupted agricultural environments because of warfare on the Eastern Front during World War II (12). These outbreaks have often been associated with a broad spectrum of tularemia syndromes, including high proportions of cases with pleuropneumonic and typhoidal presentations. It is unclear, however, whether these cases arose from inhalation or ingestion exposures, or both. Since we specifically sought cases of glandular or ulceroglandular disease, we may have missed cases with other tularemia syndromes and underestimated the extent of the outbreak. Interviews with patients and their families did not, however, suggest an outbreak of pleuropneumonic or typhoidal tularemia.

Although tularemia has rarely been reported from the Balkans, an outbreak of ulceroglandular tularemia, suspected to be associated with infected hares, was reported in central and western Bosnia in 1995, in the aftermath of warfare (WHO, personal communication). A longitudinal ecologic study of a tularemia natural focus in Croatia revealed that the focus was a meadow-field type and that the common vole was a crucial member of the tularemia biocenosis there (19). A report of a large series of cases of tularemia in Turkey, thought to be secondary to drinking of contaminated water, showed that most patients had pharyngitis and cervical adenitis, similar to the cases in Kosovo (20,21).

Based on the findings of the investigation, general recommendations were made to improve epidemiologic surveillance, provide health education, establish improved water and waste management systems, and strengthen the public health and water and sanitation infrastructure. Educational materials were developed for health professionals and the public that described tularemia, its diagnosis and treatment, and the need for improved sanitation and hygiene, especially rodent control, protection of food and water from rodents and rodent waste, and cooking and boiling food and water. Training and materials were provided to develop a microbiology laboratory capable of diagnosing tularemia. The outbreak highlighted the need for policies that would lead to improved community water sources and waste management throughout Kosovo.

Dr. Reintjes heads the Department of Hygiene, Infectious Disease Epidemiology, and Vaccinations in the Institute of Public Health in North Rhine-Westfalia, Germany. His research interests include infectious disease epidemiology, surveillance, and methodologic research.

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Address for correspondence: Ralf Reintjes, University of Applied Sciences Hamburg, Lohbruegger Kirchstrasse 65, D-21033 Hamburg, Germany; fax: 49-40-428912704; e-mail: ReintjesR@hotmail.com

African Trypanosomiasis in Travelers Returning to the United Kingdom

David A.J. Moore,* Mark Edwards,* Rod Escombe,* Dan Agranoff,* J. Wendi Bailey,† S. Bertel Squire,† and Peter L. Chiodini*

Two returning safari tourists with African trypanosomiasis were admitted to the Hospital for Tropical Diseases, London, in a 3-day period, compared with six cases in the previous 14 years. We describe the clinical features, diagnosis, and problems encountered in accessing appropriate therapy, and discuss the potential for emergence of this disease in increasingly adventurous international travelers.

A 51-year-old man returned from a 14-day game-viewing vacation in the Luangwa Valley of southern Zambia on October 11, 2000. He had been well while traveling but had sustained numerous mosquito and tsetse fly bites. Two days after his return, he noticed an enlarging, slightly tender, erythematous lesion on his right shoulder (Figure 1a). Two days later he became very ill with severe generalized myalgia, abdominal discomfort, diarrhea, vomiting, headache, fever, rigors, and sweats, but did not seek medical attention. On day 10 after his return, he consulted his primary-care physician and was admitted to his local hospital. No malaria parasites were seen on a blood film, but numerous trypomastigotes of *Trypanosoma* sp. were identified, confirming the diagnosis of African trypanosomiasis. The patient was transferred to the Hospital for Tropical Diseases, London. The trypomastigotes of *T. b. rhodesiense* are indistinguishable morphologically from those of *T. b. gambiense*, but from the epidemiology of these infections the patient was presumed to have *T. b. rhodesiense* infection, for which the recommended initial therapy is intravenous suramin.

Two days later the hospital admitted a second returning traveler with *T. brucei* (also presumed to be *rhodesiense)* infection, in this case acquired during a 14-day safari vacation to Kenya and Tanzania. This 30-year-old male patient visited the Ngorongoro Crater, Serengeti National Park, and Lake Manyara (Figure 2), but noted tsetse bites only while in the Serengeti, 7 days before symptom onset. He reported a 2-day history of an enlarging, painless, nonpruritic skin lesion on his neck, intermittent fever, a single rigor, and two brief episodes

*Hospital for Tropical Diseases, London, United Kingdom; and †Liverpool School of Tropical Medicine, Liverpool, United Kingdom

of diarrhea and vomiting. A characteristic trypanosomal chancre was present on the skin of the submandibular region (Figure 1b). The second patient also received intravenous suramin.

Both patients exhibited a rapidly rising systemic inflammatory response (initial C-reactive protein values 235 and 185 mg/L, respectively; normal <5 mg/L), lymphopenia (nadir counts 0.1 and 0.42 x 10^9/L; normal range [NR] 1.5-4.0 x 10^9/L), thrombocytopenia (nadir counts 30 and 119 x 10^9/L; NR 150-400 x 10^9/L), and mild hyponatremia (129 and 131 mmol/L; NR 136-145 mmol/L). Neither patient had notable coagulopathy. Specific antitrypanosomal serologic testing by immunofluorescence assay (IFA) yielded the following results: patient 1: day 1 immunoglobulin (Ig) M 1:40, IgG negative; day 28 IgM 1:20, IgG 1:80; and patient 2: day 1 IgM negative, IgG 1:40; day 28 IgM 1:20, IgG 1:160. Screening dilution was 1:20. Specific antitrypanosomal IFA was negative for all cerebrospinal fluid (CSF) samples tested.

Both patients were treated in accordance with a protocol developed and used in Malawi and Uganda by workers from the Liverpool School of Tropical Medicine. Intravenous suramin (reconstituted in sterile water to a 10% solution, with the required dose added to 200 mL 5% dextrose and infused over 2 hours) is administered at doses of 5, 10, and 20 mg/kg on days 1, 3, and 5, respectively (maximum 1.5 g/dose). Close observation is needed to detect signs of an idiosyncratic (anaphpylactic-type) reaction. Some authors recommend that a test dose of 100 to 200 mg of suramin be given before the first full dose, to exclude immediate hypersensitivity. Fresh CSF samples are examined on day 5 to determine whether there is evidence of central nervous system (CNS) involvement, as defined by elevated IgM, pleocytosis, or presence of trypomastigotes. Lumbar puncture is deliberately deferred until circulating trypanosomes have been cleared, to minimize the theoretical risk of iatrogenic introduction of trypomastigotes
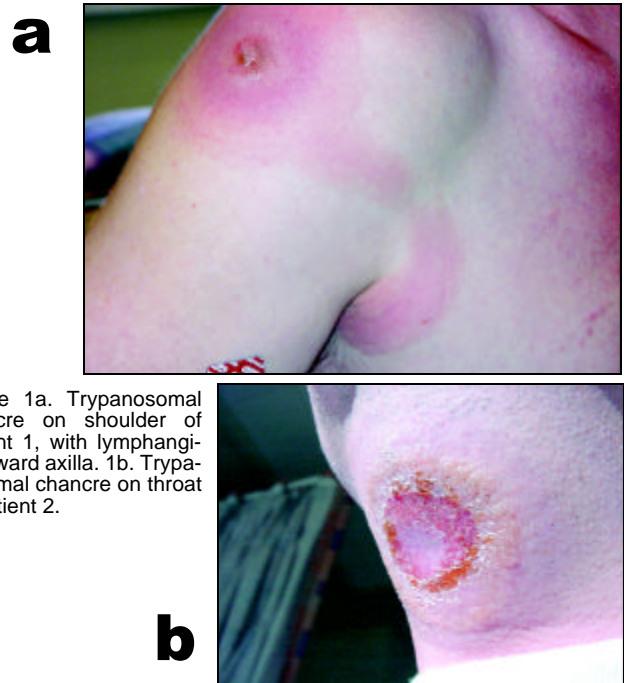
Figure 1a. Trypanosomal chancre on shoulder of patient 1, with lymphangitis toward axilla. 1b. Trypanosomal chancre on throat of patient 2.
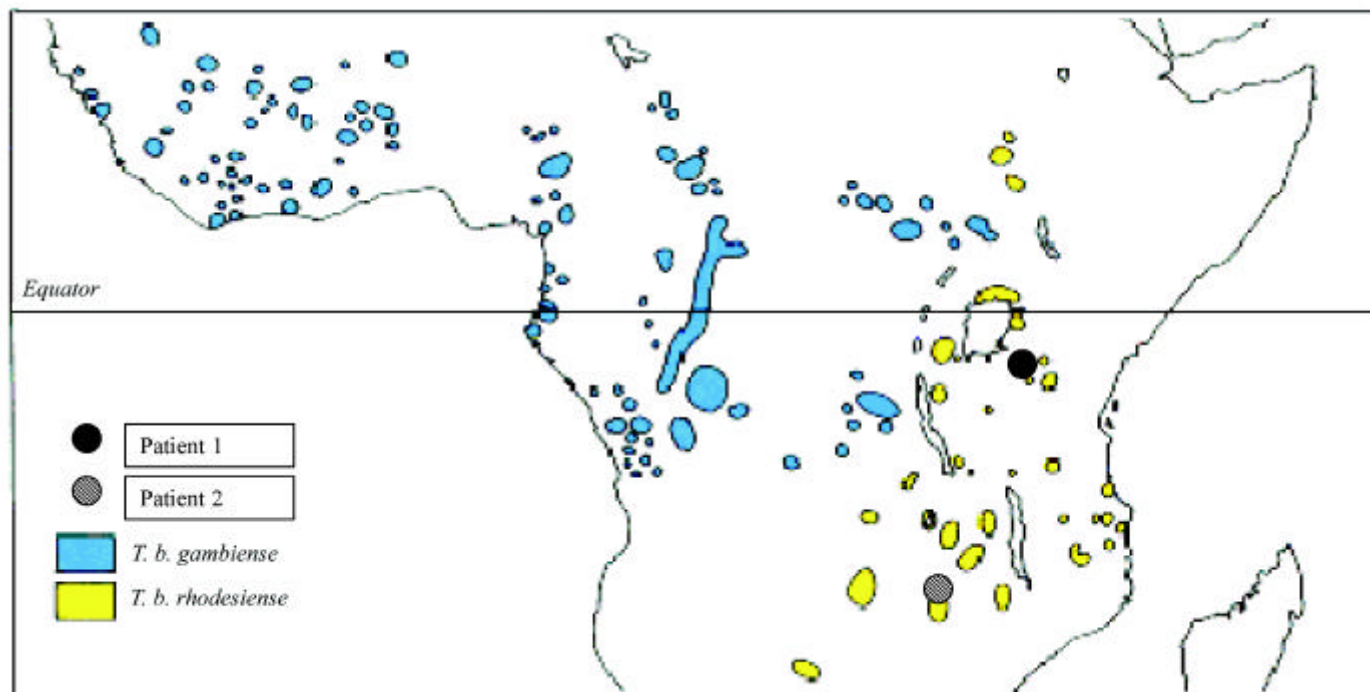
Figure 2. Map of sub-Saharan Africa, indicating itineraries of two patients with *Trypanosoma brucei rhodesiense* infection

into the CSF. This delay also aids interpretation of CSF findings as, even if there were blood in the CSF, any trypanosomes found would still indicate CNS infection. CNS involvement is treated with melarsoprol in 12 escalating doses over 4 weeks and, although an accelerated 10-day schedule for *T. b. gambiense* has recently undergone successful clinical trials in Angola (1), this regimen has not been validated for the treatment of *T. b. rhodesiense*. Suramin is ineffective in CNS disease; however, melarsoprol is a toxic arsenical compound associated in some cases with encephalopathy, which can be fatal, and is thus best avoided unless definitely required. If there is no evidence of CNS involvement, therapy with suramin (20 mg/kg intravenously, maximum 1.5 g/dose) is continued and administered on days 9, 16, 23, and 30.

Both our patients received the full 7 doses of suramin, as neither had evidence of CNS disease on CSF examination. Interestingly, both patients described a subjective dysesthesia of the fingertips during suramin therapy, which resolved on treatment completion and was not associated with any objective evidence of neuropathy on clinical examination. Transient moderate proteinuria, a known side effect of suramin, was noted in both patients and resolved after treatment.

The number of patients with imported *T. b. rhodesiense* visiting clinicians in nonendemic areas is likely to increase. For *T. b. rhodesiense*, which predominates in East Africa, wild and domestic animals are important reservoirs. Wherever people come into close proximity with these reservoirs (when visiting game parks, for example) the potential exists for transmission to humans by the tsetse fly vectors of the *Glossina morsitans* group. In contrast, for *T. b. gambiense*, which

predominates in West Africa, the human population itself is the most important reservoir and *G. palpalis* tsetse flies are the most important vector. The resurgence of sleeping sickness in Africa is mainly due to *T. b. gambiense*. Countries reporting epidemics of *T. b. gambiense* include Sudan, Angola, and the Democratic Republic of Congo, where the number of cases (certainly underreported) increased from 7,700 in 1990 to 27,044 in 1998 (2). The areas affected are not usually visited by tourists or adventure travelers. Further epidemiologic information is available on the World Health Organization website (3). In large part, the resurgence of *T. b. gambiense* is due to the breakdown of health systems in regions of civil and military unrest and is cause for considerable concern.

Imported African trypanosomiasis has hitherto been rare in the United Kingdom, with only six cases in the last 14 years at the Hospital for Tropical Diseases, most recently in 1996. Aside from the temporal proximity of the two cases described, we were concerned that both patients had acquired infection in areas commonly frequented by international safari-tourists from around the world. We know of nine subsequent additional European cases of imported African trypanosomiasis (*T. b. rhodesiense*) from Tanzania, and of an Australian who acquired the infection from the same location in 1998 (4). In 1999 Sinha et al. reported only the 20th and 21st cases of *T. b. rhodesiense* infection imported into the United States (5). However, increasing numbers of travelers are exposed to tsetse fly bites in disease-endemic areas, and many of the game animals that the safari-tourist will view, including bushbuck, waterbuck, and lion, are potential reservoirs. Furthermore, with increases in tsetse fly activity reported in many regions of sub-

Saharan Africa (including the Luangwa Valley), the potential for further cases is substantial. Neither of the locations from which our patients acquired their infections is novel, but the sudden appearance of two cases in such a short time is noteworthy. Clinicians should thus be alerted to the possibility of an increase in cases of imported African trypanosomiasis in safari-tourists.

Next, we emphasize three key diagnostic points. First, diagnosis in the two cases described was straightforward because, as is often the case with early presentations of *T. b. rhodesiense*, the patients had classic chancres and parasites were easily identified in the blood. Had the patients delayed in seeking medical attention or been exposed to *T. b. gambiense*, identifying parasites in the blood would have been more difficult, leaving the risk of disease progression to neurologic involvement before the diagnosis could be confirmed. Second, when CSF samples are taken, either to confirm or exclude late-stage neurologic trypanosomiasis, they must be examined in the laboratory within 20 minutes of the sample's being drawn. After this time, the parasites are likely to lyse spontaneously and be missed. Third, serology is not required for the diagnosis of *T. b. rhodesiense* in blood, as the levels of parasitemia are high. Furthermore, the number of *T. b. rhodesiense* clones circulating in East Africa has led to problems with test sensitivity, and the specificity is not well characterized. Serology, however, can aid the decision as to whether there is CNS involvement.

Finally, we emphasize the difficulties that we encountered (on a Saturday) in securing a supply of the therapeutic agent. Suramin was available neither from our own pharmacy (the last batch had been discarded after its expiration date passed 2 years earlier) nor from eight other regional tropical or infectious disease units in the United Kingdom, France, and Belgium. Previous use of suramin in oncology prompted us to contact a large regional oncology unit in London, but the drug was not available. Initial inquiries of hospital pharmacies in Liverpool was not promising. However, a supply was finally located at the Liverpool School of Tropical Medicine, and 3 doses, sufficient for 5 days of treatment, were sent to London by courier. The rest of the drug, obtained from the Centers for Disease Control and Prevention, arrived on day 5 after admission. The Hospital for Tropical Diseases has since secured a supply of suramin from the manufacturer in Germany, which we have in turn provided to another hospital for treatment of another patient.

There is grave concern about the international availability of a number of antiparasitic drugs, with considerable uncertainty about the sustainability of supplies of several, including suramin and melarsoprol, which manufacturers deem commercially unattractive. As the current epidemic of trypanosomiasis continues, access to effective therapies must be ensured in disease-endemic areas. Although drug resistance is potentially a serious problem, ensuring access to the currently available therapies poses a considerable challenge, which ultimately may depend on governmental or World Health Organization intervention. This issue has also been highlighted recently elsewhere (6).

A logistical problem in nonendemic areas, where imported trypanosomiasis is likely to remain rare, is access to agents such as suramin, for which the demand is small and which are therefore very seldom stocked by general hospital pharmacies but are needed urgently when a patient is admitted. Fortunately, our first patient was relatively well and not adversely affected by the 8-hour delay in initiation of therapy, but this might not always be the case. It is vital that a mechanism is in place to allow physicians working in nonendemic areas who are involved in the management of patients from trypanosomiasis-endemic areas to have access to suramin and several other essential but rarely used agents (such as melarsoprol, eflornithine, nifurtimox, and benznidazole; the last two drugs used for treatment of South American trypanosomiasis) from a central repository 24 hours a day, 7 days a week.

Dr. Moore is specialist registrar in tropical medicine at the Hospital for Tropical Diseases, London, and was previously an HIV research fellow at the Kobler Center, Chelsea and Westminster Hospital, London.

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Address for correspondence: Peter L. Chiodini, Department of Clinical Parasitology, The Hospital for Tropical Diseases, Mortimer Market, Capper Street, London WC1E 6AU, United Kingdom; fax: 44-207-383-0041; e-mail: peter.chiodini@uclh.org

High Rates of Tuberculosis in End-Stage Renal Failure: The Impact of International Migration

David A.J. Moore, Liz Lightstone, Babak Javid, and Jon S. Friedland

We studied a cohort of patients requiring renal dialysis who had migrated to the United Kingdom from tuberculosis (TB)-endemic countries and found extremely high rates of TB (1,187 cases per 100,000 per year), partly associated with end-stage diabetic renal disease. We recommend enhanced vigilance and screening of such patients, both to reduce illness and death and to prevent nosocomial spread of TB among susceptible persons.

I mmigrants, asylum seekers, and refugees from war and oppression often originate in countries where tuberculosis (TB) is endemic and relocate to more affluent countries, where the incidence of TB is low. A proportion of such persons will have or develop renal failure requiring renal replacement therapy (hemodialysis or continuous ambulatory peritoneal dialysis [CAPD]). Chronic renal failure impairs immune function and is associated with an increased incidence of TB. Among patients with chronic renal failure requiring renal replacement therapy, rates of TB 10- to 25-fold greater than those in the general population have been reported from the United States, Canada, Europe, and Japan, equating to incidence rates of approximately 250 cases per 100,000 per year (1-3). In areas of TB endemicity, the relative risk attributable to chronic renal failure requiring dialysis appears to be similar (4), although as the background rate in these settings is so much greater the absolute rate will be considerably higher. The impact of international migration and the increase in asylum seekers and refugees on the incidence of TB in renal dialysis patients has not been previously explored.

The Study

We investigated the incidence of TB in patients referred for dialysis over a 5-year period (April 1, 1994, to March 31, 1999) to the Hammersmith Hospital, situated in an area of London that is home to many immigrants, refugees, and asylum seekers. A mean of 156 patients underwent dialysis in the

Imperial College of Science, Technology and Medicine, Hammersmith Hospital, London, United Kingdom

Hammersmith Dialysis Unit each year of the study; a mean of 45 patients were new to the program each year. Patients in the renal replacement therapy cohort database who had been diagnosed with and treated for TB were identified by manual searching of hospital microbiology and histopathology databases and the handwritten logbook in which all local reports to the U.K. Communicable Disease Surveillance Centre are recorded. Duration of follow-up under the care of the renal unit (within the specified 5-year study period) was calculated for each patient. The length of time that patients had been resident in the United Kingdom was not available. Eleven TB cases (two fatal; the remainder making a complete recovery following treatment) were identified from 431 dialysis patients, including 191 who received peritoneal dialysis (CAPD). The incidence of TB was 1,187 cases per 100,000 renal patients per year. Case notes for all but one patient diagnosed with TB were available for review.

Patients had been on dialysis for 24.5 ±8.5 months (± standard error of the mean). During the study period, the total dialysis population at the Hammersmith Hospital comprised 41% Caucasians, 40% Asians, 18% black Caribbeans, and 1% from other parts of the world, including six from the Indian subcontinent, three from sub-Saharan Africa, and one from the Republic of Ireland. All the TB patients were born overseas. During the period of study, patients were not consistently screened for TB at time of entry into the United Kingdom. Specific screening of renal failure patients was also not routine, and no patient in our group received any form of chemoprophylaxis. In general, purified protein derivative (PPD) testing is not routine in the United Kingdom, where most of the population has had BCG vaccination or exposure to infection. PPD responses are less useful in the renal failure population, who tend to be anergic. Two of our patients did have PPD tests during diagnostic workup; results were negative.

Patients were treated with standard quadruple therapy of isoniazid, rifampicin, pyrazinamide, and ethambutol for 2 months; isoniazid and rifampicin treatment continued for 4 to 10 months thereafter. Once a fully sensitive pathogen had been cultured, ethambutol was discontinued. Nine of the 11 TB patients had fully drug-sensitive organisms. For the other two, diagnosis was histologic only, but complete cure followed standard therapy. However, there were some delays in diagnosis, and one patient died before the diagnosis was confirmed by culture of *Mycobacterium tuberculosis* from peritoneal fluid. Despite this, infection did not develop in any staff or other patient contacts found by contact tracing. In four (40%) of the TB patients, the underlying cause of renal failure was diabetes mellitus, although only 19% of the total dialysis population had diabetes (13% of Caucasians, 24% Asians, and 19% Afro-Caribbeans). Other causes of renal failure included hypertension, chronic pyelonephritis, immunoglobulin A nephropathy, and HIV nephropathy (only one of six patients tested was HIV seropositive). When first seen, six patients had pulmonary or pleural TB, including one patient with empyema. Four patients had peritoneal, two lymph node, and one hepatic TB. Of the

six patients with pulmonary TB, one also had peritoneal TB and one had concomitant lymph-node infection. No evidence suggested an outbreak of TB, and no clustering of cases occurred (three cases occurred in 1994, two in 1995, one in 1996, three in 1997, and two in 1998).

Conclusions

Our study showed that the incidence of TB in patients requiring dialysis is extremely high and 100-fold greater than the incidence of TB in the general population of England and Wales, which was 12 per 100,000 in 1998 (5). These figures are probably an underestimate of the extent of TB in renal disease since ascertainment was based on definitive microbiologic or histologic confirmation of diagnosis and legally required reports. We cannot exclude the possibility that occasional patients were treated on the basis of clinical suspicion and not reported. Nor did we evaluate TB cases in dialysis-independent patients with renal failure.

Part of the explanation for our findings is that patients came from countries where TB is endemic. However, TB incidence rates in England and Wales from 1988 to 1998 were, at the most, 210 and 132 per 100,000 population among black African patients and patients from the Indian subcontinent, respectively (5), figures which are substantially less than in our renal replacement therapy patients. Two other factors likely to contribute to the high rates of TB are renal failure (1-3) and the high prevalence of diabetes mellitus in patients from the Indian subcontinent, a group in whom we have noted a high incidence of end-stage renal disease (6). Diabetes mellitus appears to be associated with TB in patients with renal failure (7). Although most disease was pulmonary, peritoneal TB in CAPD patients is relatively common, and the diagnosis should always be considered in patients with persistent conventional culture-negative peritonitis.

Two of our patients died, which may reflect the fact that the clinical symptoms of TB can be difficult to distinguish from those of uremia, causing delay in diagnosis, although dialysis-dependent renal failure has been shown to be a potent contributor to death (8). HIV infection is an infrequent cause of chronic renal failure among patients at the Hammersmith Hospital and was not a major influence on TB reactivation in this patient group. However, our data suggest that renal failure requiring renal replacement therapy is as potent a risk factor for reactivation of TB as any (including HIV infection) previously described.

There are two important messages from this study, which showed that renal failure in immigrants is associated with extremely high rates of TB in the context of migration from disease-endemic areas and a high incidence of diabetes mellitus. First, it is critical that screening for latent TB infection in patients with renal failure occur at a very early stage in people who come from areas of the world where TB is endemic, a recommendation in line with those of the American Thoracic Society, Centers for Disease Control and Prevention, and the Council of the Infectious Diseases Society of America (9). Treatment for latent TB infection should be routinely considered in such patients. Although the sensitivity of TB skin testing is substantially reduced in the setting of chronic renal failure with rates of anergy in excess of 30% (10), this should not preclude its use as a screening tool because specificity is unaltered. Second, since chronic renal failure necessitates frequent attendance at medical facilities, the potential for nosocomial spread among susceptible persons is considerable. Enhanced vigilance is necessary to ensure early diagnosis of infectious cases and prompt institution of appropriate therapy.

Dr. Moore is a fellow in Infectious Diseases, who has a special interest in tuberculosis and HIV infection.

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Address for correspondence: Jon S. Friedland, Dept. of Infectious Diseases, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Hammersmith Hospital, London W12 0NN, United Kingdom; fax: 44-208-383-3394; e-mail: j.friedland@ic.ac.uk

First Case of Human Rabies in Chile Caused by an Insectivorous Bat Virus Variant

**Myriam Favi,* Carlos A. de Mattos,† Verónica Yung,†
Evelyn Chala,‡ Luis R. López,‡
and Cecilia C. de Mattos†**

The first human rabies case in Chile since 1972 occurred in March 1996 in a patient without history of known exposure. Antigenic and genetic characterization of the rabies isolate indicated that its reservoir was the insectivorous bat *Tadarida brasiliensis*. This is the first human rabies case caused by an insectivorous bat rabies virus variant reported in Latin America.

In Latin America, rabies in bats was suspected during the 1910s in Brazil and was definitively diagnosed for the first time in Trinidad in 1931 (1,2). Since then, rabies has been diagnosed in numerous species of nonhematophagous bats throughout this region (3). Despite these early discoveries, the important role of nonhematophagous bats in the epidemiology of the disease remained overshadowed by the presence of canine and vampire bat rabies in the region (4). During the past decade, with the control of dog rabies in many urban areas and the incorporation of antigenic and molecular typing of viral variants into rabies surveillance programs, an appreciation for the importance of nonhematophagous bats in rabies epidemiology began to emerge in Latin America (5-9). Rabies virus has been isolated frequently from insectivorous and frugivorous bats in cities across Latin America (5,10-12). This situation also characterizes the current epidemiologic pattern of rabies in Chile, where dog rabies has been controlled. The last human rabies case in Chile caused by a dog bite occurred in 1972 (5); since 1985, insectivorous bats have been the main rabies reservoirs identified. As such, these bats are the most important source of infection for the sporadic rabies cases diagnosed in domestic animals every year (5). In 1996, after a period of 24 years with no known human rabies deaths, the first human rabies case with an insectivorous bat as the source of infection was reported in Chile (13).

Case Report

On February 13, 1996, a 7-year-old boy from Doñihue in Administrative Region VI was admitted to the Hospital Clínico Fusat of Rancagua in the region (Figure 1) with a 2-day history of adynamia and dizziness. On admission, the child was calm, cooperative, and afebrile. Physical examination revealed anisocoria, ptosis of the left upper eyelid, and strabismus. There was no sensory loss, but ambulatory difficulties and abundant sialorrhea were observed. Brain computerized axial tomography (CAT) scan was normal. Polyradiculoneuritis was suspected, and gamma globulin was administered intravenously. The presumptive clinical diagnosis was encephalitis. On February 15, progressive paralysis developed that evolved to respiratory failure; the boy was connected to a mechanical ventilator. The patient could still follow simple orders. On February 18, he lapsed into a coma with severe hypotonia and total loss of reflexes. CAT scan showed diffuse cerebral edema, and the electroencephalogram indicated no electric activity. Intracranial hypertension developed, and the patient was put under hyperventilation and treated with intravenous dexamethasone, mannitol, and acyclovir.

Since a virus was considered the most probable cause, laboratory tests were conducted to determine the presence of the
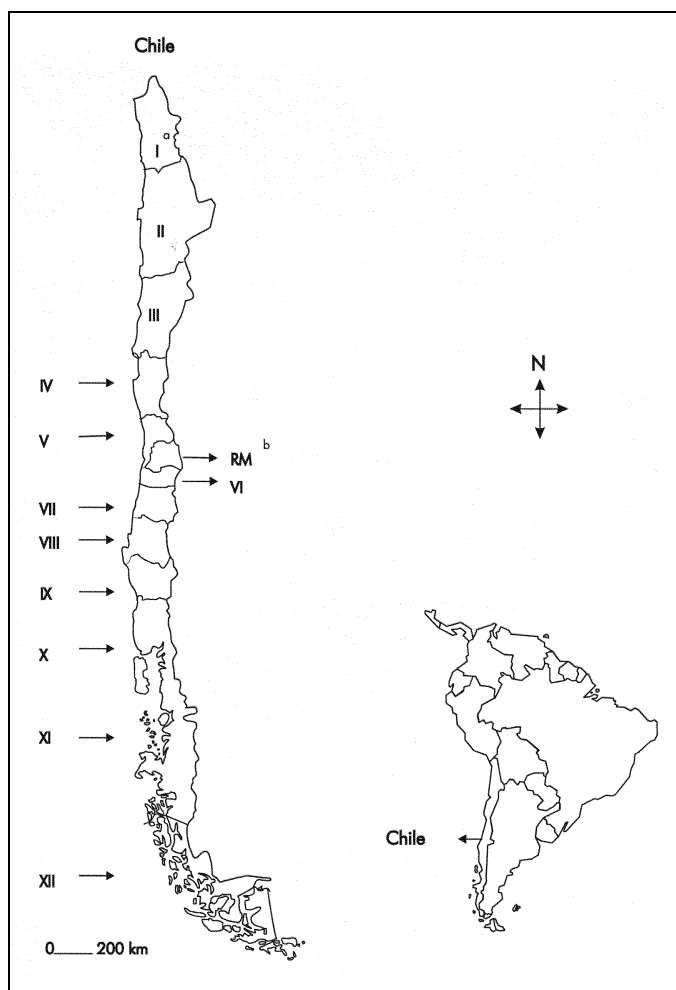
Figure 1. Map of South America showing the geographic position of Chile and map of Chile presenting the geographic distribution of the administrative regions of the country. [a]Number of the corresponding administrative region. [b]Metropolitan region.

*Instituto de Salud Pública, Ministerio de Salud Pública, Santiago, Chile; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and ‡Hospital Clínico Fusat of Rancagua, Rancagua, Chile

following viruses: herpes, measles, Coxsackie, echo, and polio. All results were negative. Interviews with relatives and the boy's nanny revealed that bats had been observed in the family's house. The nanny also reported that she had seen a bat flying away from the child's toy box. Even though these interviews failed to reveal any direct contact with bats or any history of an animal bite, this epidemiologic information prompted the physicians to suspect rabies. On February 26, 1996, a serum sample and corneal smear were obtained from the patient and sent to the Rabies Laboratory of the Instituto de Salud Pública de Santiago (ISP). A rabies antibody titer of 1:625 was found in the serum specimen by using the indirect fluorescent-antibody (IFA) technique (14). The patient had no history of rabies vaccination to account for the presence of antibodies. The corneal smear was negative for rabies antigen by the direct fluorescent-antibody (DFA) assay (15). On March 4, a second serum sample, cerebrospinal fluid, and saliva were obtained. The second serum sample was tested simultaneously with the first one by IFA assay, and a titer of 1:15,625 was detected. The cerebrospinal fluid showed a titer of 1:125. These findings confirmed the presumptive clinical diagnosis of rabies. The saliva sample was negative by DFA assay and suckling mouse inoculation (15,16).

The patient died on March 5, 1996, when artificial respiratory support was disconnected. Postmortem tissue samples of cerebral cortex, hippocampus, cerebellum, and nuchal skin biopsy were sent to the ISP Rabies Laboratory for diagnosis. The cerebellum and skin specimens were positive for rabies virus antigen by DFA assay.

Rabies postexposure prophylaxis with the suckling mouse brain Fuenzalida-Palacios vaccine was administered to the victim's mother and to 10 health-care providers who had possible contact with the patient's saliva. The rabies postexposure prophylaxis schedule used was 2 mL of vaccine, subcutaneously, on each of days 1, 2, 3, 4, 5, 6, 21, and 90. Blood samples were taken from vaccinees on day 14 after the initial dose of vaccine; IFA assay showed that adequate immune responses had developed.

The virus was isolated from the patient's brain tissue by intracerebral inoculation of suckling mice (16). To help identify the possible source of infection, the virus was antigenically and genetically characterized. Antigenic characterization of the virus was carried out by using a panel of eight monoclonal antibodies directed against the viral nucleoprotein, provided by the Centers for Disease Control and Prevention. The MAbs were used in an IFA assay as described (9,17). These analyses identified a rabies antigenic variant associated with *Tadarida brasiliensis* (free-tailed bat) in Chile, which had been designated as antigenic variant 4 (AgV4) (9,17).

Genetic characterization was done by sequencing a 320-bp portion of the rabies virus nucleoprotein gene from nucleotide position 1,157 to 1,476, as compared with the SADB 19 strain (18,19). Briefly, genomic viral RNA was extracted from infected tissue by using TRIzol (Invitrogen, San Diego, CA, formerly GIBCO-BRL Inc.) according to the manufacturer's
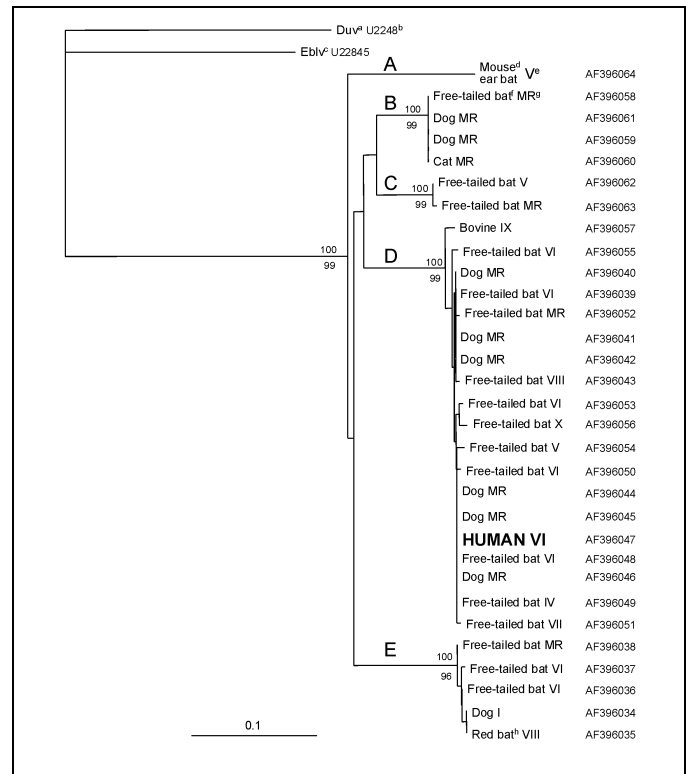

Figure 2. Neighbor-joining tree comparing the human rabies isolate with representatives of the rabies genetic variants obtained from insectivorous bats and domestic animals in Chile (18). Bootstrap values obtained from 100 resamplings of the data by using distance matrix (top) and parsimony methods (bottom) are shown at nodes corresponding to the lineages representing the rabies virus variants (A, B, C, D, and E) currently circulating in Chile. Only bootstrap values >50% are shown at the branching points. The bar at the left corner indicates 0.1 nucleotide substitutions per site. [a]Duvenhage virus, [b]GenBank accession number, [c]European bat Lyssavirus, [d]*Myotis chiloensis*, [e]Roman numerals indicate the administrative region where the sample was obtained, [f]*Tadarida brasiliensis*, [g]Metropolitan region, [h]*Lasiurus borealis*.

instructions. Complementary DNA was produced by a reverse transcription polymerase chain reaction with primers 10 g and 304 (19) and was sequenced by using the Taq Big Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol, on an Applied Biosystems 377 DNA automated sequencer (Applied Biosystems).

This human rabies virus isolate was compared with viruses obtained from domestic animals and insectivorous bats in urban centers in Chile from 1977 to 1998 (18). PileUp and Pretty programs of the Wisconsin Package, Version 10 (Genetic Computer Group, 2000, Madison, WI), were used to produce sequence alignments and comparative nucleotide analyses. The programs DNADIST (Kimura-two parameter), NEIGHBOR (Neighbor-joining method), and DNAPARS (parsimony method) from the PHYLIP package, Version 3.5 (20), were used in the phylogenetic studies. The bootstrap method, as implemented by the SEQBOOT program from PHYLIP, was followed by the use of DNADIST and NEIGHBOR for the distance matrix analyses. SEQBOOT was also used before employing DNAPARS for the parsimony studies. Graphic

representation of the trees was constructed with the TREE-VIEW program (21).

Although five genetic variants of rabies virus are found in Chile (18) (Figure 2, groups A to E), a reservoir has been identified for only two: *T. brasiliensis* (Figure 2, group D) and *Lasiurus* sp. (Figure 2, group E). Phylogenetic analyses of the Chilean human isolate demonstrated that it segregated in group D. This group represents the genetic variant of rabies virus most frequently isolated throughout the country, formed by viruses from the Metropolitan Region and Regions IV, V, VI, VII, VIII, IX, and X (Figure 1). The high bootstrap value that supports the inclusion of this virus in group D and the very close genetic relationship it has with the other members of this group (average genetic distance 0.5%) clearly show that *T. brasiliensis* is the likely reservoir of the rabies virus isolated in this case.

Conclusions

The absence of a history of an animal bite, the clinical presentation of the disease without the classic signs of hydrophobia or aerophobia, and the absence of any human rabies cases for a period of 24 years in Chile were the primary reasons that rabies was not first suspected and a definitive diagnosis was delayed in this case. Retrospective studies of human rabies epidemiology have demonstrated that it is not uncommon to observe rabies cases in which there is no history of a bite, mainly in situations involving insectivorous bat rabies variants. For example, of the 17 human rabies cases associated with insectivorous bats reported in the United States from 1980 to 1996, only one had clear documentation of a bite (22). Without proper education, patients may not be aware of the risks from a bat bite. Moreover, the wound may not be appreciated as a concern because of the limited injury inflicted by the bat's small teeth (23). Finally, there may not be an opportunity to obtain a history from a pediatric patient or to discern an exposure that occurs during sleep or other circumstances (24).

In cases in which a patient shows clinical signs of central nervous system involvement of unknown or suspected viral origin, health-care providers should be aware of the importance of conducting a thorough medical history to appropriately assess the possibility of rabies. With the important changes in the epidemiologic patterns of rabies in Latin America, this disease should be included in the differential diagnosis of neurologic diseases characterized by acute encephalitis and progressive paralysis, even when no previous history of an animal bite exists and even in regions where canine rabies has been eradicated.

Acknowledgments

Dr. Favi is the head of the Rabies Laboratory of the Instituto de Salud Pública de Chile. Her research interests include rabies diagnosis and the study of the molecular epidemiology of rabies in Chile and other Latin American countries.

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Address for correspondence: Carlos A. de Mattos, Rabies Section, Viral and Rickettsial Zoonosis Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop G33, Atlanta, GA 30333, USA; fax: 404-639-1058; e-mail: cdd9@cdc.gov

An Outbreak of Community-Acquired Foodborne Illness Caused by Methicillin-Resistant *Staphylococcus aureus*

Timothy F. Jones,* Molly E. Kellum,† Susan S. Porter,‡ Michael Bell,† and William Schaffner*§

Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) are increasingly community acquired. We investigated an outbreak in which a food handler, food specimen, and three ill patrons were culture positive for the same toxin-producing strain of MRSA. This is the first report of an outbreak of gastrointestinal illness caused by community-acquired MRSA.

I nfection with methicillin-resistant *Staphylococcus aureus* (MRSA) has been reported in the United States for over 30 years. Initially, MRSA infections were primarily a problem of hospitals and nursing homes; by 1997, 50% of health-care-acquired *S. aureus* isolates in the United States were methicillin resistant (1). Beginning in the early 1980s, cases of community-acquired MRSA were reported, primarily in persons with a history of injection drug use and other high-risk patients (2). More recently, community-acquired MRSA has been described in both adults and children who did not have extensive exposure to hospitals or other apparent risk factors (3,4). We describe the first report of a community-acquired outbreak of acute gastroenteritis caused by MRSA.

Outbreak Report

A family purchased shredded pork barbeque and coleslaw from a convenience-market delicatessen. The pork was reheated in a home microwave, and three adults ate the food 30 minutes after it was purchased. Approximately 3 to 4 hours after eating the meal, the three adults--who had not eaten another common meal together in the preceding week--had nausea, vomiting, and stomach cramps. Two children at the dinner who did not eat barbeque or coleslaw did not become ill. Two of the three ill adults were taken to a hospital for evaluation, where they were treated and released. Vomiting ceased

*Tennessee Department of Health, Nashville, Tennessee, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡West Tennessee Regional Health Department, Jackson, Tennessee, USA; and §Vanderbilt University School of Medicine, Nashville, Tennessee, USA

after treatment with phenothiazine, and nausea and cramps resolved the following day.

Methods

Ill family members were interviewed by the local health department, and an environmental inspection was performed at the market where the food was purchased. Market employees were interviewed, and stool cultures were obtained from the three ill persons. Specimens of barbequed pork and coleslaw were collected from the market, and nasopharyngeal swabs were collected for culture from three food preparers. Follow-up nasopharyngeal cultures were obtained from one ill family member 8 months after her acute illness to assess persistent carriage.

Twelve cultures of *S. aureus* recovered from stool samples of the ill family members, food specimens, and nasal swabs of the food preparers were sent to the Centers for Disease Control and Prevention for further testing. The identification of all *S. aureus* isolates was confirmed by conventional biochemical tests, and all isolates were screened for methicillin resistance by using a 1-μg oxacillin disk. Five of the 12 isolates appeared to be methicillin resistant by disk diffusion. Oxacillin susceptibility of the isolates was confirmed by broth microdilution (5) with plates read manually. All isolates were tested for staphylococcal enterotoxins by the use of a reversed passive latex agglutination test (Oxoid Ltd., Hampshire, UK). Molecular typing of all isolates was performed by pulsed-field gel electrophoresis (PFGE) with Sma I-digested chromosomal DNA. Gels were interpreted by standard criteria (6).

Results

S. aureus was recovered from the stool cultures of the three ill persons, three samples taken from the barbequed pork, one sample from the coleslaw, and five nasal swabs from three food handlers at the convenience market. Comparison of all the isolates by PFGE showed that five isolates were indistinguishable (Figure). These isolates were those from the stool cultures of three family members (A, B, and C); the coleslaw; and from the nasal swab of food preparer C. This strain produced staphylococcal enterotoxin C and was identified as being MRSA. These isolates were resistant to penicillin and oxacillin but sensitive to all other antibiotics tested. Two different strains of *S. aureus* recovered from the nasal swab of food preparer B also produced staphylococcal enterotoxin C and differed only slightly from the MRSA strain by PFGE. However, both isolates were methicillin-sensitive *S. aureus* (MSSA). These two isolates were categorized as closely related subtypes of the outbreak strain. The *S. aureus* isolate recovered from the third food preparer (A) was MSSA, produced staphylococcal enterotoxin A, and was determined by PFGE to be unrelated to the outbreak strain. Although the *S. aureus* isolates from the samples of pork barbeque each produced staphylococcal enterotoxin C, they were MSSA and unrelated to the outbreak strain by PFGE.
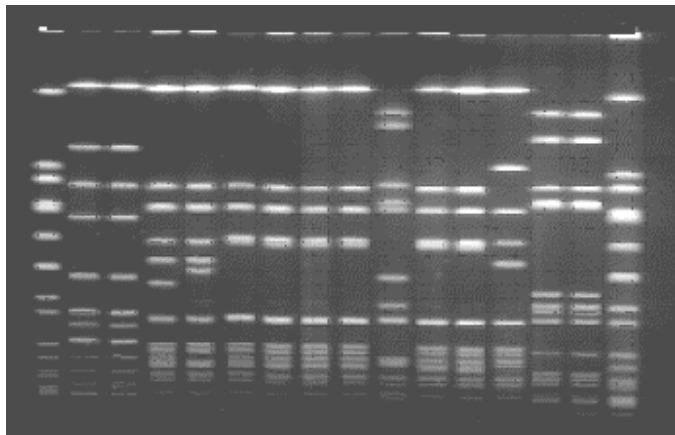
Figure. Pulsed-field gel electrophoresis profiles of Sma I-digested chromosomal DNA. Lanes 1 and 16, NCTC 8325 standard; lane 2 and 3, methicillin-sensitive *Staphylococcus aureus* (MSSA) nasal isolates from food preparer A; lanes 4 and 5, MSSA nasal isolates from food preparer B; lane 6, methicillin-resistant *S. aureus* nasal isolate from food preparer C; lane 7, MRSA stool isolate from family member A; lane 8, MRSA stool isolate from family member B; lane 9, MRSA stool isolate from family member C; lanes 10 and 11, MSSA follow-up isolates from family member C; lane 12, MRSA isolate from slaw; lanes 13, 14, and 15, MSSA isolates from barbequed pork.

The barbequed pork and coleslaw were prepared at the store where they were purchased. An environmental inspection of the facility performed after the outbreak revealed no apparent lapses in technique or procedure that would have contributed to the outbreak. No additional cases of illness related to this outbreak were reported to the local health department.

Food handler C, who was carrying the outbreak strain of MRSA, performed various tasks at the store, including preparing foods and handling barbecued pork and coleslaw. She reported no recent gastrointestinal illness nor chronic health problems, history of admission to a hospital, or use of antibiotics in the previous 6 months. She also denied close contact with persons who lived or worked in health-care facilities or other group settings. She did, however, visit an elderly relative, who resided in a nursing home, approximately 2 to 3 times each month before the outbreak. She reported that this person had a staphylococcal infection and had subsequently died. The employee refused to identify her relative, and further medical information or isolates from that person were not available.

A follow-up nasopharyngeal culture was collected from family member C approximately 8 months after her acute illness. This culture was positive for two different strains of MSSA, but not MRSA. One isolate was indistinguishable by PFGE from that of the MRSA strain isolated from the same patient during the outbreak. This isolate produced enterotoxin C, as did the strain of MRSA she was previously carrying. Polymerase chain reaction testing of this isolate confirmed that it carried the mecA gene, suggesting that the original MRSA strain had reverted to MSSA by loss of a regulatory region. The other isolate, which produced enterotoxin D, was determined by PFGE to be unrelated to any of the other strains previously seen in this investigation.

Conclusions

Despite its ubiquity as a health-care-acquired pathogen and increasing reports of community-acquired infections, MRSA has not previously been reported as a cause of outbreaks of gastroenteritis. Staphylococcal food poisoning is estimated to account for 185,000 foodborne illnesses per year in the United States; most of these go unreported (7). Because staphylococcal food poisoning is toxin mediated and generally self-limited, antibiotics are not used for therapy. Also, many *S. aureus* isolates obtained as part of outbreak investigations may not be tested for antibiotic susceptibility, and therefore methicillin-resistant strains may go unrecognized as the cause of foodborne outbreaks of acute gastroenteritis. Methicillin-resistant strains of *S. aureus* are as likely to produce enterotoxins as are methicillin-sensitive strains. Therefore, outbreaks of acute gastroenteritis due to MRSA are not unexpected.

Until recently, MRSA has been considered primarily a health-care-associated pathogen, causing invasive disease in which multidrug resistance poses a substantial challenge to successful treatment. Food has been implicated as a source of spread in one outbreak of blood and wound infections in hospitalized immunocompromised patients (8). There has been some debate about the appropriateness of the term "community-acquired" to describe many *S. aureus* infections in which distant hospital exposures cannot be excluded with certainty and colonization can persist for years. In this outbreak, it appears that MRSA-contaminated food was the vehicle in a cluster of illnesses affecting low-risk persons within the community and that this food was likely contaminated by an asymptomatic carrier whose only apparent exposures were intermittent visits to a nursing home. This outbreak could be an example of second-generation spread of a health-care-associated pathogen into the community. The outbreak strain of MRSA, however, was resistant only to penicillin and oxacillin and was sensitive to all other antibiotics tested. A strain originating in a health-care facility would have an increased likelihood of being multidrug resistant (9).

MRSA was isolated from a food handler involved in this outbreak, and an MSSA strain with an identical PFGE pattern was subsequently isolated. The strain obtained on follow-up retained the mec-A gene and probably represents a genetic mutation in a regulatory region. Shortly after the outbreak, another food handler was carrying two strains of MSSA with a PFGE pattern very similar to the outbreak strain of MRSA. These strains appear to be related to the outbreak strain but with loss of the mec-A determinant. The existence of multiple strains of *S. aureus* in persons involved in this outbreak is not surprising, as 20% to 40% of adults are estimated to be colonized at any time, and multiple strains may be present in the same person. Colonization with MRSA carries a greater risk for infection than does colonization with MSSA (10).

This outbreak suggests that as MRSA becomes increasingly common in the community, it will be implicated in expressions of all the clinical manifestations of staphylococcal

infection. While antibiotic-resistant strains are not expected to be clinically more virulent or challenging in the setting of acute outbreaks of gastroenteritis, MRSA may cause soft-tissue and other infections in the community that are difficult to treat. This episode demonstrates the spread of MRSA into the community and is a harbinger of the increasing impact of health-care-associated organisms in settings and among populations previously considered to be unthreatened by this emerging antimicrobial-resistant pathogen.

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Dr. Jones is deputy state epidemiologist and director of the FoodNet Program in the Tennessee Department of Health.

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Address for correspondence: Timothy Jones, Communicable and Environmental Disease Services, Tennessee Department of Health 4th Floor, Cordell Hull Bldg., 425 5th Ave. N. Nashville, TN 37247, USA; fax: 615-741-3857; e-mail: tjones4@mail.state.tn.us

Human Infection with *Cryptosporidium felis*: Case Report and Literature Review

Simone Cacciò,* Elena Pinter,† Rosanna Fantini,† Ivano Mezzaroma,† and Edoardo Pozio*

An infection with *Cryptosporidium felis* in an HIV-positive man from Italy was successfully treated with paromomycin, despite the patient's having a CD4+ cell count of 31/mm^3. Fourteen cases of human infection with *C. felis* have been described, all in the past 3 years, emphasizing the public health importance of *Cryptosporidium* parasites other than *C. parvum*.

Parasites belonging to the genus *Cryptosporidium* are an important and widespread cause of enteric disease in humans and in many other vertebrates. The most commonly identified etiologic agent of human cryptosporidiosis is *Cryptosporidium parvum* (1), which, based on the molecular characterization of oocysts, can be divided into two genetically distinct subpopulations: genotype 1 (or the "anthroponotic genotype"), which is associated exclusively with human infection; and genotype 2 (the "zoonotic genotype"), which is associated with both human and animal infection (2). For many years, *C. parvum* was considered to be the only etiologic agent of human infection. However, the use of molecular tools with a greater capacity to detect and differentiate strains has resulted in the identification of other human pathogens: *C. felis*, *C. meleagridis*, the *C. parvum* dog genotype, and possibly *C. muris* (2).

In Italy, all *Cryptosporidium* oocysts detected in immunocompetent and immunosuppressed persons have been identified as *C. parvum* (3). In this report, we describe the first Italian case of a human *C. felis* infection, which occurred in an HIV-positive man who, in spite of a very low CD4+ cell count, successfully recovered with paromomycin treatment.

The Study

On December 12, 2000, a 42-year-old homosexual man with severe diarrhea was admitted as an outpatient to the Umberto I° University Medical Center in Rome, Italy. The man had been diagnosed with *Hepatitis B virus* infection in 1982 and with HIV infection in 1988. In 1989, his CD4+ cell count had reached 189/mm^3, and he started antiretroviral therapy with zidovudine. He was later treated with all nucleoside analogs (individually or combined), and in 1996 he began treatment with highly active antiretroviral therapy (HAART).

The specific HAART regimen was changed several times because of his poor immunologic and virologic response. In the course of years, the man had many episodes of oral candidiasis, which were responsive to fluconazole therapy. In July 2000, he weighed 87 kg and had a CD4+cell count of 52/mm^3; at that time, HAART was changed to include ritonavir, indinavir, stavudine, and didanosine. In November 2000, the patient had isolated episodes of diarrhea, but feces were not investigated for the presence of parasites. At the beginning of December 2000, he was affected by impetigo, which was treated with erythrocine (1800 mg/day). Two days after beginning that therapy, he began to have up to 10 episodes of diarrhea per day. Erythrocine was interrupted after 3 days, and, in the period of 1 week, the patient lost 9 kg. Stool specimens were tested for a wide panel of enteric pathogens (bacteria, viruses, helminths, and protozoa, including microsporidia). The parasitologic examination of stools showed *Cryptosporidium* oocysts (3x10^6 oocysts/mL of feces). The oocyst diameter was in the range of 4.5-4.9 μm. Oocysts reacted strongly with two monoclonal antibodies conjugated with fluorescein (MeriFluor, Meridian Diagnostics, Cincinnati, OH; *Cryptosporidium* Immunofluorescence Test, Microgen Bioproducts Ltd, UK). No other pathogen was found in the specimens. The patient was treated with paromomycin (1 g, 3 times/day). On the second day of treatment, the diarrhea promptly resolved, decreasing from 10 to 2 bouts per day. Paromomycin treatment was continued until mid-February (CD4+ cell count 31/mm^3) without further diarrheal episodes, and stools were negative for *Cryptosporidium*.

DNA was extracted from the whole feces according to the FastPrep method of da Silva et al. (4), and the diagnostic fragment of the small subunit ribosomal RNA (ssu-rRNA) was amplified by polymerase chain reaction (PCR) with the primer set CPBDIAGF and CPBDIAGR (5). The sequence of the PCR product was determined, and a comparison with all ssu-rRNA *Cryptosporidium* sequences available in databanks revealed 100% similarity with the homologous fragment of *C. felis* (accession number AF087577). To obtain additional information on the nature of the species, a PCR-restriction fragment length polymorphism assay (primer set cry9 and cry15) (6) that targets a fragment of a *Cryptosporidium* oocyst wall protein gene was used (7). This second analysis confirmed the identification of *C. felis* (Figure).

To the best of our knowledge, this is the only case of a *C. felis* infection for which the clinical course and the response to therapy have been reported. Although the literature contains numerous reports of paromomycin treatment of human *Cryptosporidium* infection, the results regarding the efficacy of paromomycin are contrasting, possibly because it was always assumed that the etiologic agent was *C. parvum*. The current knowledge that several species and genotypes can infect humans suggests that the efficacy of paromomycin could depend on the specific *Cryptosporidium* species/genotype and its susceptibility to this drug. In our case report, the infected person had a very low CD4+ count, which has been considered as

*Istituto Superiore di Sanità, Rome, Italy; and †University of Rome La Sapienza, Rome, Italy
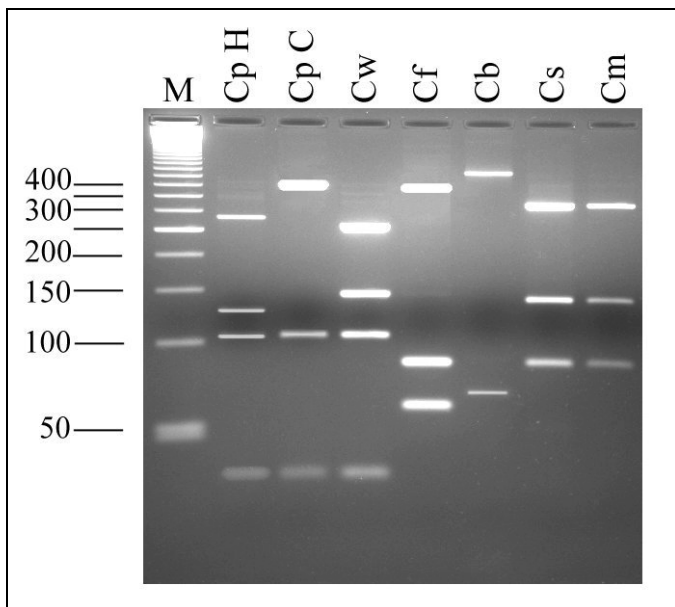
Figure. Electrophoretic separation of *Cryptosporidium* oocyst wall protein gene-polymerase chain reaction products digested with the endonuclease *Rsa*I. Lane M, 50-bp size ladder; CpH, *Cryptosporidium parvum* human genotype; CpC *C. parvum* calf genotype; Cw, *C. wrairi*; Cf, *C. felis*; Cb, *C. baileyi*; Cs, *C. serpentis*; Cm, *C. muris*.

one of the most important factors in the failure of paromomycin treatment (8,9). The concomitance of the erythrocine treatment and severe watery diarrhea suggests that the drug had altered the intestinal flora and, in turn, favored the growth of the parasite. A concomitant influence of paromomycin treatment and the interruption of erythrocine treatment can be also postulated.

There have been 14 cases of human infections with *C. felis* reported; all have occurred in the past 3 years (10-13). These cases occurred in North and South America, Africa, and Europe, and they involved both immunocompetent (n=4) and immunosuppressed (n=10) persons.

There have also been cases of human cryptosporidiosis in which cats were identified as the source for human infection, yet the species of *Cryptosporidium* remained unknown. Glaser et al. (14) examined the association between *Cryptosporidium* infection and animal exposure in HIV-infected persons and concluded that only dog ownership presents a risk, although minimal; no significant risk was associated with cat ownership. However, cats have been successfully experimentally infected with *C. parvum* oocysts of human and bovine origin, and a *C. felis* infection of a cow has been demonstrated (15). These data show not only that the host specificity of some of the *Cryptosporidium* species that infect mammals is less restricted than previously thought but also that there is a complex circulation of species in the environment. Under such circumstances, it is often difficult to trace the source of an infection. In our case, the *C. felis*-infected person did not have a cat at home, but the city where he lives (Rome) is home to a plethora of stray and domestic cats (approximately 0.1 cat per inhabitant). Infection may have occurred upon accidental contact with

oocysts in the environment. The public health importance of *Cryptosporidium* parasites other than *C. parvum* needs to be assessed.

Dr. Cacciò is a molecular biologist at the Laboratory of Parasitology of the Istituto Superiore di Sanità in Rome, Italy. His work focuses on the characterization of the enteric protozoa *Cryptosporidium* and *Giardia*.

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Address for correspondence: Edoardo Pozio, Laboratory of Parasitology, Istituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy; fax: 39 06 4938 7065; e-mail: pozio@iss.it

Human Herpesvirus 6-Associated Hemophagocytic Syndrome in a Healthy Adult

Hiroshi Tanaka, Tetsuo Nishimura, Mikiko Hakui, Hisashi Sugimoto, Keiko Tanaka-Taya, and Koichi Yamanishi

Virus-associated hemophagocytic syndrome is a fulminant disorder associated with systemic viral infection and characterized pathologically by multiple-organ infiltration of hemophagocytic histiocytes into the lymphoreticular tissues. This is the first report of a previously healthy adult in whom *Human herpesvirus 6* reactivation induced this syndrome with severe hemodynamic and respiratory distress.

Virus-associated hemophagocytic syndrome (VAHS) is a fulminant disorder associated with systemic viral infection and characterized pathologically by multiple-organ infiltration of hemophagocytic histiocytes into the lymphoreticular tissues. VAHS has been associated with *Epstein-Barr virus* (EBV), cytomegalovirus (CMV), adenovirus, and *Herpes simplex virus* (HSV), as well as with a variety of nonviral infections (1). There have been several reports of VAHS in children caused by *Human herpesvirus 6* (HHV-6) infection. We report what may be the first record of reactivated HHV-6 causing VAHS with severe hemodynamic and respiratory distress in a previously healthy adult.

Case Report

A healthy 22-year-old man with a high fever lost consciousness and was admitted to our hospital. On admission, a skin rash covered his whole body; cervical, axial, inguinal, and supraclavicular lymphadenopathy and hepatosplenomegaly were observed. The leukocyte count was 14,590/mm^3, the hemoglobin concentration 13.9 g/dL, and platelet count 12.7x10^4/mm^3. Elevated liver enzymes (glutamate oxalacetic transaminase 155 U/L, glutamate pyruvate transaminase 379 U/L) were found, along with elevated lactate dehydrogenase 911 U/L (normal 130-290 U/L) and C-reactive protein 4.9 mg/dL (normal <0.2 mg/dL). Serum antibody tests for CMV and HSV were negative, but a serum antibody test for EBV was positive (1:640) on day 2 after admission. Infectious mononucleosis was suspected, and the case was managed conservatively without antibiotics for 13 days after admission.

On day 14, the patient suddenly went into shock and severe respiratory distress developed, with PaO$_2$ 45 mmHg and

Osaka University Medical School, Osaka, Japan

PaCO$_2$ 35 mmHg at FiO$_2$ of 100%. Pancytopenia was evident, with a leukocyte count of 270/mm^3, hemoglobin of 9.1 g/dL, and platelet count of 9.7x10^4/mm^3 (Figure 1). The patient's bone marrow was hypocellular, with a nucleated cell count of 1.6x10^4/mm^3 (normal 13.7-23.1x10^4/mm^3), and showed an increased number of histiocytes with hemophagocytosis and mature large granulolymphocytes. Elevated serum concentrations of tumor necrosis factor (TNF)-alpha (44 pg/mL; normal <15.6 pg/mL), interleukin (IL)-1^2 (129 pg/mL; normal <3.9 pg/mL), IL-6 (3,415 pg/mL; normal <3.1 pg/mL), IL-8 (15,598 pg/mL; normal <31.2 pg/mL), and granulocyte-colony stimulating factor (G-CSF) (165,000 pg/mL; normal <39.1 pg/mL) were observed. Blood and organ bacteria cultures were negative. The CD4/CD8 ratio (0.73; normal 0.88-1.84) was low, and complete suppression of immunoglobulin was observed, with decreased immunoglobulin (Ig) A (30 mg/dL; normal 115-440 mg/dL), IgG (620 mg/dL; normal 1,000-2,060 mg/dL), and CD19 (1.1%; normal 9.7-17.3 %). Serum antibody tests for HHV-6 were positive (1:80 on day 7 and 1:280 on day 30 after admission), suggesting that this was a case of HHV-6 reactivation. HHV-6B was isolated as previously described (2) from peripheral blood mononuclear cells (PBMC) on day 5 after admission (Figure 2), suggesting VAHS induced by HHV-6.

On day 14 after admission, at the onset of pancytopenia, subcutaneous administration of recombinant human G-CSF (Lenograstim; Chugai Pharmaceutical Co. LTD., Tokyo, Japan) was started at 2 µg/kg. Improvement in hematologic parameters was apparent 8 days after the start of G-CSF, and
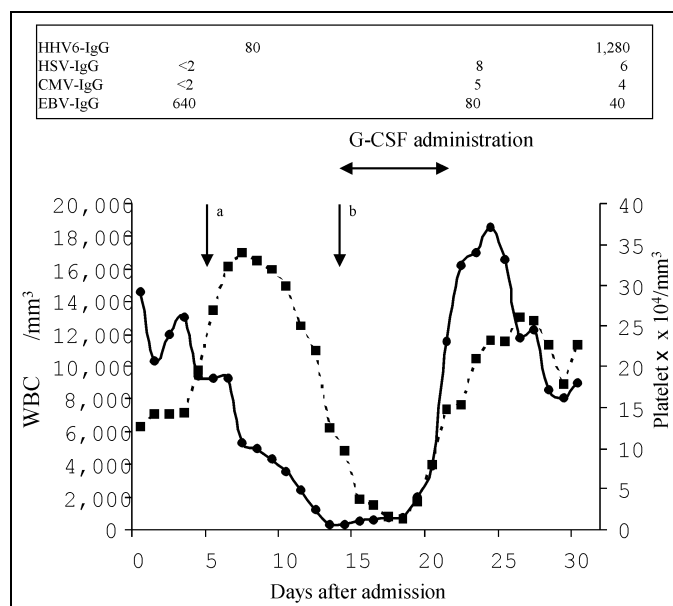
Figure 1. Changes in leukocyte count (solid circles), platelet count (solid squares), and serum antibodies tests for *Human herpesvirus 6* (HHV-6), *Herpes simplex virus* (HSV), cytomegalovirus (CMV), and *Epstein-Barr virus* (EBV) after admission. Values in the box demonstrate immunoreactivity to HHV6, HSV, CMV, and EBV. [a]HHV-6B was isolated from peripheral blood mononuclear cells on day 5 after admission. [b]Pancytopenia was diagnosed on the day 14 after admission. IgG = immunoglobulin G; G-CSF = granulocyte-colony stimulating factor (G-CSF).
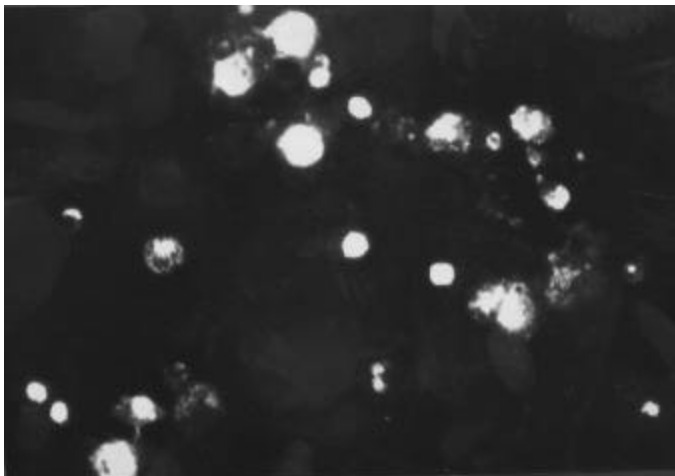
Figure 2. Immunofluorescence micrograph of peripheral blood mononuclear cells infected with *Human herpesvirus 6B* isolated on day 5 after admission.

there were no further complications (Figure 1). With mechanical ventilation and fluid resuscitation with catecholamine, the respiratory and hemodynamic status improved. HHV-6B was not isolated from PBMC on day 32 after admission. The symptoms and signs of VAHS disappeared completely, and the patient was discharged 44 days after admission.

Conclusions

VAHS is characterized by prominent phagocytosis of erythrocytes and nucleated blood cells in the bone marrow and lymph nodes. The general symptoms are fever and hepatosplenomegaly. Some cases have been been associated with hypercytokinemia by TNF-alpha, IL-1-beta, and interferon (IFN-gamma), resulting in severe hemodynamic collapse and acute lung injury (3). Lymphocyte activation induces excessive production of IFN-gamma, which acts on a variety of cells, resulting in macrophage activation and tissue damage. In keeping with this proposed injury mechanism, successful treatment with cyclosporin A has been documented (3). We administered G-CSF alone, although the serum G-CSF concentration was markedly increased when VAHS was diagnosed, suggesting remarkable up-regulation. HHV-6 is a lymphotropic virus that grows in PBMC. It is widespread in the normal population; >80% of the general population in Japan is seropositive. Exanthema subitum has been considered a manifestation of primary infection with HHV-6 (2). There have been several reports of VAHS in children caused by HHV-6 infection (4,5). The few adults who escape HHV-6 infection during childhood and acquire primary HHV-6 infection as young adults have a self-limited, febrile illness, usually associated with lymphadenopathy and resembling infectious mononucleosis (6). To our knowledge, this is the first report of a healthy adult in whom HHV-6 reactivation induced VAHS with severe hemodynamic and respiratory distress.

Dr. Tanaka is a trauma surgeon at the Department of Traumatology and Acute Critical Care Medicine of the Osaka University Medical School, Osaka, Japan. His current interest is leukocyte function in patients with severe trauma.

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Address for correspondence: Hiroshi Tanaka, Department of Traumatology, Osaka University Medical School, 2-15, Yamadaoka, Suita-shi, Osaka, 565-0871, Japan; fax: 81-6-6879-5720; e-mail: tanaka@hp-emerg.med.osaka-u.ac.jp

Presence of *Rickettsia felis* in the Cat Flea from Southwestern Europe[1]

**Francisco J. Márquez,* Miguel A. Muniain,†
Jesús M. Pérez,* and Jerónimo Pachón‡**

Rickettsia felis, formerly called ELB agent, was identified by using molecular biology techniques in the cat flea (*Ctenocephalides felis felis*) from southwestern Spain. For the first time this flea-transmitted rickettsia has been detected within its vector in Eurasia.

Members of the genus *Rickettsia* are commonly associated with hematophagous arthropods such as ticks, fleas, or lice. *Rickettsia felis*, formerly ELB agent, was detected in 1990 when tissues from the cat flea, *Ctenocephalides felis,* were examined under electron microscopy. After this, several antigenic and molecular studies concerning this rickettsia were developed (1). *R. felis* is maintained in cat fleas by transovarian transmission (2). Infection in humans has been described in the USA (3), Mexico (4), and Brazil (1) by polymerase chain reaction (PCR) amplification and recently in France by serologic tests (1).

During a study concerning rickettsial organisms transmitted by ticks in southwest Spain, using molecular tools for diagnosis, a rickettsial microorganism was detected in some cat fleas on domestic cats and dogs from different counties of the Cadiz Province.

The Study

The fleas used in this study (60 females and 11 males) were collected, together with ticks, from 2 cats and 12 dogs from eight localities of Cadiz Province in southwestern Spain from May to August of 1999 (Figure, Table). The hosts were domestic and peridomesticated dogs and cats living in a range of health-care conditions. Collected fleas were fixed in 70% ethyl alcohol and stored at 4°C until they were processed. Taxonomic determination was made by using current taxonomic keys (5,6). All specimens subjected to analysis were *C. felis felis* (Bouché, 1835).

DNA was extracted from 14 lots of fleas (ranging from 1 to 11 specimens per lot) by using the DNeasy_Tissue kit(Qiagen GmbH, Hilden, Germany) (7). Elution of DNA was made in

*Universidad de Jaén, Jaén, Spain; †Hospital Universitario Virgen Macarena, Seville, Spain; and ‡Hospital Universitario Virgen del Rocío, Seville, Spain

Figure. Localization of randomly selected sampling points in Cadiz Province for cat fleas with indication of the presence of *Rickettsia felis* by polymerase chain reaction procedures. Localities are 1. San Roque, 2. Tarifa, 3. Vejer de la Frontera, 4. Benalup, 5. Jerez, 6. Arcos de la Frontera, 7. San José del Valle, 8. Paterna de Rivera.

100 µL of TE buffer (1 mM Tris HCl, 0.1 mM EDTA). Extraction blanks, consisting of water processed along with flea samples, were also included as controls.

A Biometra DNA Thermalcycler (Gottingen, Germany) was used for all PCR amplification. Three microliters of each DNA extraction were added to 27 µL of master mixture for each reaction. Final reagent concentration was 0.2 µM for each primer, 200 µM for each deoxynucleotide triphosphate (Promega Corp., Madison, WI), 2 U of Biotaq polymerase (BioLine, London, UK), and 1x Bioline buffer. The following thermal cycler parameters were used with the primer pairs for citrate synthase (*glta*) RpCS.877p and RpCS1258n (8), 120-kDa genus common antigen (*ompB*) (rfompbf: 5'–GAC AAT TAA TAT CGG TGA CGG, and rfompbr: 5'-TGC ATC AGC ATT ACC GCT TGC), 190-kDa protein antigen (*ompA*) Rr190.70p, and Rr190.602n (8): 96°C (90 sec), followed by 35 cycles of 94°C (30 sec), 50°C (30 sec), and 72°C (45 sec), followed by an extension period (72°C, 7 min). For the amplification of a 426-base pair fragment of 16S rRNA gene, we used the primers fD1 (9) and Rc16S.452n (10) and 59°C as annealing temperature.

Seven lots from five localities around Cadiz and Gibraltar bays were positive to amplification of fragments of 16S rRNA, *glta*, *ompA* and *ompB* genes.

In brief, after amplification, primers and nucleotides were removed from 300 µL of PCR products by purification on the Wizard PCR preps purification system (Promega, Madison,

Table. Origin, host range, lot composition, and polymerase chain reaction results of cat fleas (*Ctenocephalides felis felis*), captured in Cadiz Province, southwestern Spain, used in this study

Locality	Capture date	Host	*C. f. felis* females	*C.f. felis* males	PCR result
Arcos de la Frontera	23/05/1999	dog	2	1	+
Benalup	09/08/1999	dog	7	4	+
Jerez	24/05/1999	dog	2	-	-
Paterna de Rivera	24/05/1999	dog	5	1	-
San José del Valle	24/05/1999	dog	6	-	-
San Roque	21/05/1999	dog	8	-	+
San Roque	21/05/1999	dog	1	-	+
Tarifa	11/06/1999	dog	5	3	+
Tarifa	11/06/1999	dog	1	-	+
Vejer de la Frontera	12/06/1999	dog	3	2	-
Vejer de la Frontera	09/08/1999	cat	7	-	+
Vejer de la Frontera	09/08/1999	cat	4	-	-
Vejer de la Frontera	09/08/1999	dog	5	-	-
Vejer de la Frontera	09/08/1999	dog	4	-	-

WI). Approximately 100 fmol of the purified PCR product (4-5 µL) were used directly in the sequencing reaction.

The PCR cycle sequencing was performed for each amplicon by using the correct forward or reverse primers and the Silver sequence DNA Sequencing System (Promega). Sequencing reaction products were loaded twice on 40 cm 6% polyacrylamide 7M urea gels by electrophoresis in the Sequi-Gen Nucleic Acid Sequencing System (BioRad, Hercules, CA) at 55 W of constant electrophoresis (55°C) and separated for 4 hr 30 min. and 2 hr 30 min, respectively. Gel was silver stained by using the standard Promega protocol. A permanent record was made in scanning the gel. To determine the sequence of positions near primers, we used a 10% polyacrylamide 7M urea electrophoresis gel. The sequence of both strings was determined twice for each fragment.

Sequences obtained were compared with those from other *Rickettsia* species in GenBank by using the BLAST utility (National Center for Biotechnology Information, Bethesda, MD) and FASTA routine from GCG environment. Fragment sequence for 16S rRNA, *glta*, *ompA*, and *ompB* sequence were identical to previously reported sequence for *R. felis*. The 16S rRNA amplified fragment was identical to previously reported

sequence (GenBank L28944) between positions 1 and 410 (3). The fragment sequenced for citrate synthase corresponded to positions 757 and 1138 in GenBank accession AF210692 (1). The fragment amplified for *ompA* corresponded to positions 478 to 987 in GenBank accession AF191026 (11). The fragment amplified for *ompB* corresponded to positions 599 to 1259 in GenBank accession AF210695 (1). Amplification was unsuccessful in all negative controls.

Conclusions

R. felis has been found extensively in commercial colonies and natural cat fleas, parasitizing a large range of mammalian hosts in several states of the United States (12,13).

For the first time *R. felis* was detected in Eurasia, by means of PCR and partial sequencing of genes classically used in rickettsial molecular characterization and phylogeny. The sequences of *glta*, *ompA*, *ompB*, and 16S rRNA from Cadiz cat fleas were identical to the homologous sequences previously reported for *R. felis* obtained from fleas reared in EL Laboratories (Soquel, CA) (3) and Louisiana State University (11) and isolated by Flea Data Inc. (Freeville, NY) (1).

In humans, *R. felis* may produce a clinical syndrome similar to murine typhus (3). Thus, *R. felis* could be implicated in murine typhus-compatible cases detected in southwest Spain (14), especially since the oriental rat flea, *Xenopsylla cheopis* (Rothschild, 1903), is absent from this area.

Thirteen species of flea belonging to the genus *Ctenocephalides* have been described to date (15), mainly distributed in continental Africa (16), with a worldwide contemporary distribution in a large range of hosts, mainly anthropic species of the group (*C. felis*), which has a large potential host range. The primary source of the bacterium might be Africa, where this flea genus apparently originated.

Dr. Márquez is associate professor of zoology and parasitology at the Faculty of Experimental Sciences at Jaén University, Spain. His research interests focus on medical entomology and arthropod-borne diseases.

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Address for correspondence: Francisco J. Márquez, Departamento de Biología Animal, Biología Vegetal y Ecología, Universidad de Jaén, Paraje Las Lagunillas s/n, 23071 Jaén, Spain; fax: 34-53-012141; e-mail: jmarquez@ujaen.es

Antimicrobial Sensitivity in Enterobacteria from AIDS Patients, Zambia

**James Mwansa,* Kabanga Mutela,* Isaac Zulu,*†
Beatrice Amadi,* and Paul Kelly*†**

Enterobacteria contribute to two serious clinical syndromes seen in African AIDS patients: diarrhea and septicemia. In West Africa, prophylaxis with sulfamethoxazole-trimethoprim (SXT) reduced illnesses. We report reduced sensitivity of enterobacteria to available antimicrobial agents in Zambia, with only 22% of nontyphoidal salmonellae and 6% of shigellae sensitive to SXT.

D iarrhea and septicemia, two of the most important clinical problems of African AIDS patients, are both associated with high rates of illness and death. Treatment with antimicrobial agents may play an important role in reducing illness and possibly death. Chemoprophylaxis (for example, with cotrimoxazole or sulfamethoxazole-trimethoprim [SXT]) has been shown to be effective in reducing illness and death (1,2). The evidence base relating to patterns of antimicrobial resistance in Africa is small, and antimicrobial agents are often chosen on the basis of availability and expense. Some evidence indicates that resistance patterns vary across Africa, with resistance to SXT in nontyphoidal salmonellae of 14% in Abidjan and 83% in Malawi (3). We report the prevalence of infection with three major enterobacteria in Zambian adults and children with AIDS, followed by an analysis of antimicrobial sensitivity patterns.

The Study

To determine the prevalence of infection in adults and children, cultures were performed by standard techniques on fecal samples from several groups of adults and children, all residents of Lusaka, the capital of Zambia. Group A was 124 adults and 105 children, all HIV-seropositive patients with persistent diarrhea, studied from 1995 to 1999 during three clinical trials of antiprotozoal or nutritional therapies. The average number of samples tested was 2.1 for each adult and 2.8 for each child. Group B was 216 adults enrolled in a longitudinal study of intestinal infection in a cohort of adults in a representative urban community in Lusaka; this group was studied to provide an estimate of asymptomatic carriage rates.

*University of Zambia School of Medicine, University Teaching Hospital, Lusaka, Zambia; and †St. Bartholomew's & Royal London School of Medicine, London, United Kingdom

To define the profile of antimicrobial sensitivity, isolates from Group A were analyzed together with additional isolates of nontyphoidal salmonellae, *Shigella dysenteriae*, and *S. flexneri* from a third source (Group C): routine stool or blood cultures from AIDS patients in the University Teaching Hospital from 1995 to 1999. All isolates were cultured and tested for antimicrobial susceptibility by using standard antimicrobial discs (Oxoid Ltd, Basingstoke, UK) on Mueller-Hinton agar. Zones of growth inhibition were compared with standard tables (4), and control organisms of known sensitivity were tested beside clinical isolates for verification.

Of 124 adults with persistent diarrhea in Group A, 6 (5%) were infected with nontyphoidal *Salmonella* spp. and 9 (7%) with *S. flexneri* or *S. dysenteriae*. Of 105 children with persistent diarrhea, also in Group A, 21 (20%) were infected with nontyphoidal *Salmonella* spp. and 3 (3%) with *S. flexneri* or *S. dysenteriae*. In Group B, 7 (4%) of 174 adults had one or more fecal samples positive for nontyphoidal *Salmonella* spp. in one year (1999), and 10 (6%) had one or more positive for *S. flexneri* or *S. dysenteriae*. As each adult submitted samples approximately monthly (for a total of 1,440 samples), the point prevalence in these asymptomatic adults was <1% for either infection.

Studies of patients with HIV-related persistent diarrhea in other countries in Africa have found the prevalence of enterobacterial infection to be higher. In Rwanda and Kenya, prevalences of nontyphoidal salmonellae were 11% and 16%, respectively, and of shigellae were 22% and 4%, respectively (5,6). In recent years, HIV seroprevalence in Lusaka has been estimated to be 22% to 30% (7), and the overall rate of HIV-related diarrhea is high (8).

Conclusions

Antimicrobial sensitivity patterns indicate that resistance is a substantial problem among enterobacteria in Lusaka (Table). The isolates we tested came from a tertiary hospital, which may have resulted in some selection bias, as treatment failures may be overrepresented in such patients. However, only a few bacteria tested were sensitive to SXT, in marked contrast to data from West Africa and more closely resembling the situation in Malawi (3). This level of resistance may compromise the usefulness of SXT in preventing bacterial infection in HIV-infected persons, although any effect in preventing *Pneumocystis carinii* pneumonia or isosporiasis would be valuable. Emergence of resistance to SXT was noted in San Francisco after its widespread use as prophylaxis against pneumocystosis (9). For treatment of infection with these enterobacteria in Zambia, only the more expensive antimicrobial agents now seem to be reliable. Providing effective, affordable parenteral antimicrobial agents for the efficient treatment of septicemic infection in hospitals and health centers is likely to be difficult. As clinical response sometimes occurs even when susceptibility testing in vitro suggests that the antimicrobial agent used is ineffective, controlled clinical trials are needed for these infections in different geographic regions of Africa.

Table. Summary of antimicrobial sensitivity patterns for three enterobacteria isolated from patients with HIV-related persistent diarrhea in Zambia

Antimicrobial agent[a]	No. sensitive (%) Nontyphoidal salmonellae	Shigella flexneri	S. dysenteriae
Tetracycline	37 (6)	2 (6)	3 (16)
Chloramphenicol	36 (77)	7 (22)	8 (48)
Gentamicin	119 (75)	24 (77)	18 (95)
Sulphamethoxazole-trimethoprim	25 (22)	3 (10)	0 (0)
Amoxycillin	74 (48)	9 (30)	7 (37)
Amoxycillin-clavulanic acid	95 (60)	27 (87)	12 (63)
Cephalexin	105 (66)	23 (74)	17 (89)
Cefuroxime	93 (59)	11 (35)	16 (74)
Cefotaxime	149 (88)	28 (90)	19 (95)
Nalidixic acid	107 (67)	31 (100)	19 (100)
Ciprofloxacin	157 (99)	30 (97)	18 (95)
Erythromycin	22 (14)	0 (0)	4 (21)
Azithromycin	64 (92)	9 (100)	19 (100)

[a]158 isolates of nontyphoidal salmonellae, 31 isolates of S. flexneri, and 19 isolates of S. dysenteriae were tested against all these antimicrobial agents except for azithromycin, against which 69, 9, and 19 isolates were tested respectively.

Antimicrobial sensitivity appeared to decrease from 1995 to 1999, when these isolates were being collected. For example, over this period gentamicin resistance increased from 0% to 32% in S. flexneri and from 0% to 34% in nontyphoidal salmonellae (p<0.001). In S. flexneri, cefuroxime resistance increased from 22% to 88% and cephalexin resistance from 18% to 42% over the same period (p = 0.001). The scale of use of cephalosporins in the community did not suggest that selection pressure for resistance was likely to be high. Mechanisms of resistance to cephalosporins include reduced permeability and modification of penicillin-binding protein, and emergence appears to be rapid.

Which antimicrobial agents could be recommended for treatment of bacteremic nontyphoidal salmonellosis? The most reliable results are likely to be obtained with fluoroquinolones or azithromycin, but these are expensive and their availability is limited. Gentamicin or chloramphenicol are less expensive and would be acceptable alternatives, although the probability of adverse effects is greater. Unfortunately, few antimicrobial compounds that are likely to be effective and affordable could also be given easily and safely to AIDS patients in primary and secondary care settings in Zambia. As the epidemic in Zambia enters its third decade, the situation is likely to worsen if no action is taken. We have been able to reduce antimicrobial resistance in *Vibrio cholerae* by instituting a policy of rotating the recommended antimicrobial agents during epidemics, thereby prolonging the useful life of affordable antimicrobial agents (J. Mwansa, unpub. obs.). This strategy could be extended to enterobacterial infection in AIDS. We are also considering clinical trials with combinations of antimicrobial drugs to treat these clinical syndromes in AIDS patients. As antimicrobial sensitivity patterns seem to vary across Africa, it may be difficult to generalize the results of clinical trials from one part of the continent to another.

Dr. Mwansa is a consultant microbiologist in the University Teaching Hospital, Lusaka, Zambia. He advises on control of infectious disease for the Ministry of Health and has particular interests in salmonellosis and meningitis.

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Address for correspondence: Paul Kelly, Department of Adult and Paediatric Gastroenterology, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD, United Kingdom; fax: 44-207-882-7192; e-mail: mpkelly@mds.qmw.ac.uk

Absence of Mycoplasma Contamination in the Anthrax Vaccine

Mary Kate Hart,* Richard A. Del Giudice,† and George W. Korch, Jr.*

Mycoplasma contamination of the licensed anthrax vaccine administered to military personnel has been suggested as a possible cause of Persian Gulf illness. Vaccine samples tested by nonmilitary laboratories were negative for viable mycoplasma and mycoplasma DNA and did not support its survival. Mycoplasma contamination of anthrax vaccine should not be considered a possible cause of illness.

A nthrax Vaccine Adsorbed (AVA, BioPort Corporation, Lansing, MI) is a licensed vaccine for anthrax that was administered to approximately 150,000 U.S. military personnel during the Persian Gulf War. It was used more recently as part of a comprehensive vaccination policy for Department of Defense (DOD) service members. The vaccine, which is administered subcutaneously over an 18-month schedule, is derived from sterile, acellular filtrates of microaerophilic cultures of the avirulent, nonencapsulated V770-NP1-R strain of *Bacillus anthracis*. The cultures are grown in a sterile synthetic liquid medium devoid of enriched supplements such as serum. The filtrates predominantly contain the protective antigen of anthrax. The final vaccine formulation contains protective antigen adsorbed to aluminum hydroxide (<2.4 mg/0.5 mL) as an adjuvant. Formaldehyde (<0.02%) is added as a stabilizer and benzethonium chloride (0.0025%) as a preservative. The final product is checked for potency and safety according to U.S. Food and Drug Administration (FDA) regulations.

Approximately 1.9 million doses of AVA were administered in the United States from January 1990 through August 2000 (1), with 1,544 adverse reactions (0.08% of all injections) reported through the Vaccine Adverse Event Reporting System (2). Most of these adverse reactions were limited to the injection site; local hypersensitivity, edema, and pain were the most commonly reported, although 76 (4.9%) of the adverse reactions were classified as serious.

*United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA; and †National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland, USA

The Study

Recently, Nicolson et al. (3) suggested that contamination of this vaccine with *Mycoplasma fermentans* could have been responsible for human illness specifically associated with Persian Gulf syndrome. Mycoplasma contamination is considered to be a potential problem for vaccines that are produced in cell cultures. Such vaccines have to meet specific FDA testing requirements to demonstrate the absence of mycoplasma contamination, which is considered unlikely in vaccines that are not cell culture-derived, such as AVA. However, in response to the continuing controversy surrounding this vaccine, the possibility of its being contaminated with mycoplasma was examined by two different techniques.

AVA was administered to U.S. personnel during Operation Desert Storm/Desert Shield (ODS/DS), including lots FAV 001 through FAV 007. Although the lots tested in this study were not administered to U.S. personnel deployed during ODS/DS, the filling and potency testing of lot FAV 008 in March 1991 were contemporaneous with that of FAV 007 and were subject to the same regulatory criteria for lot release and use. Its use in the current vaccination program was permitted through shelf life extension based on satisfactory potency testing.

Twenty vials of AVA, including samples from four lots, were obtained from eight DOD vaccination clinics across the United States (Table). The tested lots represented all unexpired vaccine available to the DOD at the time of testing. Testing of unexpired material was deemed necessary to avoid potential uncertainty regarding degradation of vaccine components or analytes.

These vials were divided into two matched sets and were sent to two nonmilitary laboratories for testing for mycoplasma contamination. One set was tested by culture techniques for the presence of live organisms at the Mycoplasma Laboratory, Science Applications International Corporation, National Cancer Institute, Frederick Research and Development Center, Frederick, MD. A commercial nongovernment facility, Charles River Tektagen, tested the second set for mycoplasma DNA by poly-

Table. Anthrax Vaccine Adsorbed samples evaluated for mycoplasma contamination

Vaccine lot Expiration date	Sites providing vaccine[a]
FAV048B 13 Apr 2002	355 Medical Squadron, Davis Monthan Air Force Base, AZ N.R.C., Kansas City, MO Camp Pendleton USMC
FAV047 8 Sep 2001	USAMRIID, Ft Detrick , MD (four vials) Pearl Harbor NMC
FAV031 6 Oct 2000	Ft. Worth Base Naval Clinic Pentagon Clinic
FAV008 5 Aug 2000	Davis Monthan Air Force Base, AZ 169th FW/Base Supply, McEntire ANG Station, Eastover, SC

[a]Vaccine vials were shipped to Fort Detrick on cold packs. Two vials from each lot were sent unless noted. Two vials of lot FAV047 from USAMRIID were sent to each testing laboratory as a control.

merase chain reaction (PCR) assay.

Ten anthrax vaccine vials were tested for the presence of mycoplasma by standardized cultivation methods (4-6) with three different media. The media used for isolation were SP-4 (4), DM-1 (5), and M-CMRL (6); all three supported the growth of *Mycoplasma* organisms. Vaccine samples were removed under Biosafety Level-2 conditions and tested both undiluted and at a 10-fold dilution to reduce the concentrations of stabilizer and preservative. Each culture plate was inoculated with 0.1 mL of vaccine. M-CMRL plates were incubated aerobically at 36°C, and SP-4 and DM-1 plates were incubated anaerobically at 36°C. After 10 days, all plates were examined microscopically. No evidence of *Mycoplasma* colonies was found in any of the culture plates inoculated with the AVA samples.

To determine if *Mycoplasma* organisms could survive in the vaccine, which contains formaldehyde and benzethonium chloride, a fifth lot of vaccine (FAV038) was obtained and evaluated. This vaccine lot also tested negative. To test the ability of *Mycoplasma* to survive in the vaccine, *M. fermentans* strain incognitus (7) was inoculated at a concentration of 1.54×10^8 CFU per 0.1 mL into 5 mL of vaccine and mixed. Eight serial 10-fold dilutions of this "time 0" sample were immediately plated on SP-4 agar plates. The remaining vaccine-*Mycoplasma* mixture was held at 4°C and sampled at 24, 48, and 72 hours. Tenfold serial dilutions of these sequential samples were immediately plated as described for the time 0 sample.

All SP-4 plates were incubated for a minimum of 10 days and examined for viable colonies. The colony growth on the time 0 plates was atypical, with much debris on the low dilution plates. The titer of mycoplasma from the time 0 sample was 4.2×10^5 CFU per 0.1 mL. Inactivation of the organisms by the preservatives in the vaccine was rapid, as no growth was detected on plates inoculated with samples taken from the mycoplasma-vaccine mixtures held at 4°C for 24, 48, or 72 hours.

Testing for the presence of mycoplasma DNA in the second set of AVA samples, representing four lots, was performed by a commercial testing facility (Charles River Tektagen, Malvern, PA) that used the Detection of Mycoplasma by PCR kit (#90-100K, American Type Culture Collection, Rockville, MD). Samples were tested first for the presence of any mycoplasma DNA; the species was then determined for samples that tested positive. The vaccine samples were centrifuged (12,000 x *g* for 10 minutes at 4°C) and processed for DNA extraction according to the kit's instructions. Samples were amplified in a nested PCR reaction and examined by gel electrophoresis. The positive control samples were *M. pirum* and *Acholeplasma laidlawii*, and the negative control samples were mycoplasma broth, Hut78 cell extract, and sterile RNase-free water. All 10 of the tested AVA samples were negative for the presence of mycoplasma DNA (Figure).

Conclusions

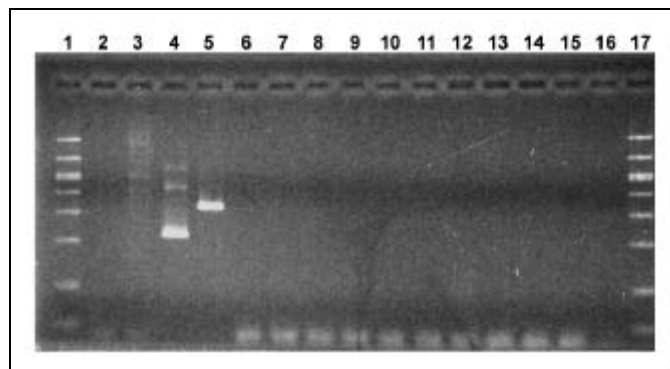No evidence was found either from culture or mycoplasma-

Figure. Evaluation of Anthrax Vaccine Adsorbed (AVA) for amplified mycoplasma DNA by gel electrophoresis. Molecular weight markers were run in lanes 1 and 17. Control samples in lanes 2-5 were mycoplasma broth, Hut 78 cell extract, *Acholeplasma laidlawii*, and *Mycoplasma pirum*, respectively. The AVA samples were in lanes 6 to 15: Lot FAV048B from Davis Monthan AFB (lane 6), Kansas City MO NRC (lane 7), and Camp Pendleton (lane 8); Lot FAV047 from Fort Detrick (lanes 9 and 11) and Pearl Harbor NMC (lane 10); Lot FAV031 from Fort Worth Base Naval Clinic (lane 12) and the Pentagon Clinic (lane 13); and Lot FAV008 from Davis Monthan AFB (lane 14) and McEntire ANG Station (lane 15). Lane 16 contained water. Bands seen below 100 base pairs are primer multimers.

specific nucleic acid amplification methods to suggest that a mycoplasma contaminant was present in the vaccine lots tested. These results are consistent with those of a previous report that found no evidence of such contamination in another anthrax vaccine (8). Additionally, *Mycoplasma* organisms deliberately added to AVA did not survive for even 1 day, presumably because of the preservatives added to the vaccine formulation to retard adventitious agent growth.

These results argue against assertions that this vaccine was contaminated with *Mycoplasma* organisms and that such putative contamination contributed to human illness. This finding is consistent with a serologic study of pre- and post-Gulf War serum samples from symptomatic and asymptomatic military personnel, which found no evidence of an association between Gulf War illness and infection with *M. fermentans* (9).

Dr. Hart is chief of the Department of Cell and Host Responses in the Virology Division of the U.S. Army Medical Research Institute of Infectious Diseases, where she supervises the Cell Culture and Hybridoma Production facilities. Her research focuses on immune responses to viruses.

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Address for correspondence: George Korch, USAMRIID, 1425 Porter Street, Frederick, MD 21702, USA; fax: 301-619-4625; e-mail: george.korch@det. amedd.army.mil

Nosocomial Endocarditis Caused by *Corynebacterium amycolatum* and Other Nondiphtheriae Corynebacteria

Karen L. Knox* and Alison H. Holmes†

The nondiphtheriae corynebacteria are uncommon but increasingly recognized as agents of endocarditis in patients with underlying structural heart disease or prosthetic valves. We describe three cases of nosocomial endocarditis caused by nondiphtheriae corynebacteria, including the first reported case of *Corynebacterium amycolatum* endocarditis. These all occurred in association with indwelling intravascular devices.

T he nondiphtheriae corynebacteria, major components of the normal flora of human skin and mucous membranes, are commonly isolated from clinical specimens. As such, they are frequently dismissed as contaminants. Recently, however, the microbiologic classification of this group of organisms and their role in clinical disease are being more clearly defined (1). In particular, *Corynebacterium amycolatum*, which was first described in 1988 (2), is becoming widely recognized as an important pathogen, although it has been underreported in part because of its misidentification as *C. xerosis* (3,4), an established human pathogen.

These organisms are causes of community-acquired endocarditis in patients who have underlying structural heart disease or are immunocompromised, as well as of prosthetic-valve endocarditis. Cases in previously healthy patients are rarely described (5,6). Hospital-acquired bacterial endocarditis accounts for 7.5% to 29% of cases of endocarditis in tertiary-care hospitals and is strongly associated with infection of indwelling intravascular lines (7). Common causative organisms include *Staphylococcus aureus*, enterococci, and coagulase–negative staphylococci. *Corynebacterium* species are rarely reported as agents of hospital-acquired endocarditis.

We describe three cases of endocarditis caused by three different species of corynebacteria in our hospital within an 18-month period, all associated with indwelling intravascular devices (IID). These accounted for 3 of 10 cases of hospital-acquired endocarditis identified during this period and include the first reported case of endocarditis caused by *C. amycolatum*. Two of the *Corynebacterium* species were resistant to

*St. George's Hospital, London, United Kingdom; and †Imperial College School of Medicine, Hammersmith Hospital, London, United Kingdom

multiple antibiotics. These cases highlight the importance of the nondiphtheriae corynebacteria as emerging multiresistant nosocomial pathogens in the growing population of patients with IID.

Case Reports

Case 1

A 74-year-old woman was hospitalized with a diagnosis of antineutrophil cytoplasmic antibody (ANCA)-positive vasculitis. Her treatment included daily plasma exchanges via a right internal jugular vascular catheter that was placed on the 5th hospital day. On day 21 of admission, she had a low-grade fever that resolved after removal of the catheter, the tip of which produced a culture of diphtheroids. Blood cultures were sterile.

The patient was discharged after 1 month but was readmitted 4 weeks later for control of vasculitis. On admission she was anemic (hemoglobin 7.0 g/dL) and had persistent microscopic hematuria and elevated C-reactive protein. She had no new vasculitic manifestations and, as previously, a grade II systolic murmur was noted at the left sternal edge. She had a fever of 38°C 48 hours after admission. Four sets of blood cultures were positive for *C. amycolatum* (Table 1). Transesophageal echocardiogram (TOE) showed a large mobile vegetation on the mitral valve, with prolapse and mild to moderate mitral regurgitation. A transthoracic echocardiogram on her previous admission had shown no mitral valve abnormality. A combination of intravenous vancomycin and oral rifampicin was begun, and the fever resolved within 48 hours. The patient refused surgical intervention, and dual antibiotic therapy was continued indefinitely. A follow-up TOE at 15 months showed improvement in mitral valve features and no vegetations. The antibiotics were stopped after 16 months, and 5 months later the patient had no clinical evidence of disease recurrence.

Case 2

A 69-year-old woman with hemodialysis-dependent chronic renal failure secondary to ANCA-positive vasculitis was admitted after acute thrombosis of a GoreTex dialysis fistula in her left arm. She had required temporary vascular access for hemodialysis for the preceding 12 days. On examination she was unwell and feverish. She had a grade I systolic murmur over the cardiac apex; no previous echocardiogram was available. Two sets of blood cultures grew *C. striatum* (Table 1), as did the tip of her vascular catheter on removal. A TOE revealed a large vegetation on her mitral valve. Because of penicillin allergy, intravenous vancomycin and oral rifampicin were begun. The prosthetic arteriovenous fistula could not be removed completely, although it was considered a potential reservoir of infection. The patient had a mitral valve replacement 22 days into medical therapy, on the 29th hospital day. Her valve was completely destroyed, but on culture was sterile. Antibiotics were continued for another 15 days; however, the patient had postoperative complications and died 4 weeks later.

Table 1. Patient characteristics, heart valve affected, source of *Corynebacterium* infection and organism cultured, London

Case	Age(y)/ sex	Underlying disease process	Site of endocarditis	Associated IID[a]	Therapy	Outcome
1	74/F	ANCA + vasculitis	Native mitral valve	Vascular catheter for HD	Vancomycin i.v. + oral rifampicin, 16 months[b]	Resolved at 21 months postdiagnosis
2	69/F	ANCA + vasculitis	Native mitral valve	Gortex AV fistula, vascular catheter for HD	Vancomycin i.v. + oral rifampicin, 37 days; mitral valve replacement	Died of unrelated causes 9 weeks after diagnosis
3	53/M	Postoperative acute renal failure	Prosthetic mitral valve (Starr Edwards)	Vascular catheter for HD CVC	Vancomycin i.v. + oral rifampicin, 42 days	Died of unrelated causes 8.5 months postdiagnosis

[a]IID = indwelling intravascular device; ANCA = anti-neutrophil cytoplasmic antibody; HD = hemodialysis; AV = arteriovenous; CVC = central venous catheter.
[b]Patient refused surgery and follow-up echocardiograms.

Case 3

A 53-year-old man underwent mitral valve replacement with a Starr Edwards metal valve. His postoperative course was complicated by acute renal failure, for which he required temporary hemodialysis by vascular catheter for 36 days. The maximum time a single line was in place was 13 days. Cultures of four vascular catheter tips after their removal grew both diphtheroids and coagulase-negative staphylococci, which were considered to be normal skin flora. During this time, a low-grade fever developed that resolved after a change of lines. Blood cultures remained sterile. Serial echocardiograms over the following 2 weeks, however, showed a worsening para-prosthetic leak. Subsequently, five sets of blood cultures grew *C. jeikeium* (Table 1). The patient completed 6 weeks of intravenous vancomycin and oral rifampicin for prosthetic valve endocarditis and was well on discharge. At follow-up, TOE studies showed no further increase in the paraprosthetic leak, and the patient had no clinical evidence of relapse of infection. He died 7 months later from noninfective causes.

Discussion

Interest in the taxonomy of the nondiphtheriae corynebacteria has increased, with a resultant reclassification of earlier defined taxa and discovery of new pathogens in the group (1,8). One of the more commonly reported human infections with these organisms is infectious endocarditis. Since 1966, several case reports and reviews have described 191 cases of nondiphtheriae corynebacterial endocarditis on both prosthetic and native valves. In only six (3.1%) of these cases was association with an indwelling intravascular device specifically documented: two patients in association with chronic hemodialysis (6), one case attributed to an infected permanent pacemaker wire (9), two in connection with ventriculo-peritoneal shunts (6), and one in association with a ventriculo-atrial shunt (10). Of reports pertaining to hospital-acquired endocarditis in particular, in four case series totaling 80 cases, *Corynebacterium* species were not identified as causative agents (7,11-13). In another series, *Corynebacterium* species accounted for 1 (3.3%) of 30 cases (14), this case occurring postcardiac catheterization. A further series of 14 cases of hospital-acquired endocarditis describes 3 (21.4%) cases due to coryneform species but gives

no species identification or source of infection (15).

We have described three cases of nondiphtheriae coryne-bacterial nosocomial endocarditis associated with IID, all occurring in a single hospital, accounting for one third of 10 cases occurring in an 18-month period. These cases include the first reported case of endocarditis caused by *C. amycolatum*. Of the remaining seven cases, two were due to methicillin-resistant *S. aureus* and five to methicillin-sensitive *S. aureus*.

Since Lehmann and Neumann proposed in 1896 that bacteria morphologically resembling the diphtheria bacillus be incorporated with it into the genus *Corynebacterium*, the classification of the coryneform bacteria has been drastically altered (8). This change has, in turn, increased the difficulty of identifying these organisms, as methods that reliably differentiate related species, such as mycolic acid chromatography, gas liquid chromatography, and molecular amplification techniques, cannot easily be used in the routine laboratory setting. During 1987 to 1995, 11 new *Corynebacterium* species were described (1). Commercial identification systems need to be updated to include those species relevant in human disease. *C. amycolatum* has only recently been included in the updated API Coryne database 2.0 (4), which, as illustrated by our case, may still lead to misidentification if used alone. That there have been no previous reports of *C. amycolatum* causing endocarditis may be due to its misidentification as other nonlipophilic fermentative corynebacteria species such as *C. xerosis* and *C. minutissimum* (3,16), both of which are associated with human disease.

Published material provides useful schema for differentiating *C. amycolatum*, *C. minutissimum*, and *C. striatum* by using colonial morphology, carbohydrate assimilation tests, and sensitivity to amoxycillin and the vibriostatic compound O/129, in conjunction with the API Coryne and API 20NE systems (17). Antibiotic sensitivity patterns may support identification, with *C. amycolatum* and *C. jeikeium* generally resistant to multiple antibiotics (18). In contrast, *C. striatum*, *C. minutissimum*, and *C. xerosis* generally are sensitive to a wide range of antibiotics.

Funke et al. (1) have published guidelines for identifying the coryneform bacteria, including simple phenotypic characteristics but also recommending more complex chemotaxonomic investigations and molecular genetic analysis if

phenotypic characteristics do not differentiate between species. These guidelines are intended to facilitate the establishment of true disease associations of these organisms. In our experience, morphologic features combined with antibiogram profiles and the Coryne API allowed identification of two out of three corynebacteria (Table 2). As the colonial morphology and the Coryne API profile did not match in the isolate from Case 1, the organism was sent to our reference laboratory, where identification as *C. amycolatum* was confirmed by gas liquid chromatography (Microbial Identification System, Microbial ID, Newark, DE).

Treatment regimens described include penicillin alone, beta-lactam antibiotics or erythromycin plus gentamicin, and vancomycin. Our three patients all received dual therapy with vancomycin and rifampicin. This combination has not previously been described but was effective in two of the three patients (including the case of prosthetic valve endocarditis), who were both free from infection at least 5 months after cessation of therapy. In a review of 19 patients with prosthetic valve endocarditis due to diphtheroids (19), 4 (57.1%) of 7 patients treated with antibiotics alone were reported as cured at least 1 year posttreatment. Of 12 patients treated both medically and surgically, 7 (58.3%) were reported as having a microbiologic cure (19).

This study highlights the importance of the nondiphtheriae corynebacteria in severe human disease. Specifically, clinicians and microbiologists must be aware of the potential risk factors for nondiphtheriae corynebacterial endocarditis in the hospital setting and the danger of overlooking positive longline tip cultures as normal skin flora or contaminants. Stringent identification of clinical isolates will be required to define the role of the nondiphtheriae corynebacteria in human disease.

Acknowledgments

Dr. Knox is a specialist registrar in clinical microbiology at St. George's Hospital, London. Her research interests include nosocomial infection and antibiotic resistance.

Dr. Holmes is an infectious diseases specialist, the senior lecturer in hospital epidemiology and infection control at Imperial College London, based at the Hammersmith Hospitals Trust. She is also codirector of the International Health Unit, Imperial College, London.

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Address for correspondence: Karen L. Knox, Department of Microbiology, 1st Floor, Jenner Wing, St George's Hospital, Cranmer Terrace, Tooting, London SW17 0RE, United Kingdom; fax: 44-208-725-5694; e-mail: kknox@sghms.ac.uk

Table 2. Identification and antibiograms of the *Corynebacteria* species in three cases of endocarditis, London

Organism: gram-positive rod, nonmotile, catalase positive	Colonial morphology	Further identification	Antibiotic sensitivity pattern (Stokes plate)	
			Sensitive	Resistant
Corynebacterium amycolatum Case 1	Dry, gray[a]	Coryne API GLC[b]	Rif, Teic, Vanc	Cip, Ery, F, Gent, Pen, Trim
C. striatum Case 2	Moist, white, smooth[a]	Coryne API	Cip, Ery, F, Gent, Rif, Trim, Teic, Vanc	Pen
C. jeikeiun Case 3	Gray, nonhemolytic[a] (aerobic growth)	Coryne API	Rif, Teic, Vanc	Cip, Ery, F, Gent, Pen, Trim

[a]Horse blood agar at 37°C.
[b]API = analytical profile index; GLC = gas liquid chromatography; Cip = ciprofloxacin; Ery = erythromycin; F = fucidin; Gent = gentamicin; Pen = penicillin; Rif = rifampicin; Teic = teicoplanin; Trim = Trimethoprim; Vanc = vancomycin.

Primary Liver Abscess Caused by One Clone of *Klebsiella pneumoniae* with Two Colonial Morphotypes and Resistotypes

Po-Ren Hsueh,* Juinn-Jong Wu,† Lee-Jene Teng,*‡ Yee-Chun Chen,* Pan-Chyr Yang,* Shen-Wu Ho,* and Kwen-Tay Luh*

Two diabetic patients with primary liver abscess, who initially responded unsatisfactorily to intravenous ceftriaxone or cefoxitin treatment and had abscess drainage, were found to be infected with a single clone of *Klebsiella pneumoniae* with two different colonial morphotypes and resistotypes. Primary liver abscess caused by second-generation cephalosporin-resistant *K. pneumoniae* strains may be an emerging problem in Taiwan.

Primary liver abscess caused by a single pathogen, *Klebsiella pneumoniae*, has long been an important infectious complication in diabetic patients in Taiwan (1,2). All *K. pneumoniae* strains causing primary liver abscess have a unique antimicrobial susceptibility pattern (resistant to ampicillin, ticarcillin, and carbenicillin but susceptible to other antibiotics including all cephalosporins and aminoglycosides) (1-4). Although multidrug-resistant strains of *K. pneumoniae,* whether community acquired or nosocomial, are not uncommon in Taiwan and other countries, these isolates had never been reported as the cause of primary liver abscess (5,6).

Case Summaries

A 42-year-old man (patient A) was admitted on July 31, 2000, for a fever of 2 weeks' duration. He had had diabetes mellitus for 20 years and alcoholism for >10 years. Physical examination and abdominal echography showed hepatomegaly and a huge abscess (12 cm x 10 cm x 8 cm) over the right lobe of the liver. Laboratory tests showed leukocytosis (14,600/µL) with a left shift. After blood cultures, ceftriaxone (2 g every 12 hours) was given. A "pigtail" catheter was inserted for continuous drainage on the 5th hospital day. Abscess aspirate culture yielded *K. pneumoniae* with two colonial morphotypes (iso-

lates on trypticase soy agar plates supplemented with 5% sheep blood [BBL Microbiology Systems, Cockeysville, MD] after 24 hours of incubation in ambient air) (Figure 1) and two resistotypes (by the routine disk diffusion method). One (isolate A1) had mucoid, opaque colonies and was resistant to ampicillin but susceptible to cefazolin and cefoxitin, and the other (isolate A2) had nonmucoid, white colonies and was resistant to ampicillin, cefazolin, and cefoxitin. Both isolates were susceptible to amoxicillin-clavulanate and cefotaxime. Blood cultures were negative. Because of persistent fever, the antibiotic was changed to imipenem (500 mg every 6 hours) on the 11th hospital day. Fever subsided 3 days after imipenem administration was begun. Imipenem was continued for a total of 24 days, followed by ciprofloxacin (750 mg every 12 hours) for 3 weeks. A follow-up echography 4 months after antibiotic treatment ended showed that the abscess had disappeared.

A 66-year-old man (patient B) with diabetes mellitus was admitted on August 4, 2000, with fever and hiccups of 4 days' duration. Physical examination was unremarkable. Laboratory tests showed elevated levels of alkaline phosphatase (585 U/L; reference range 66-220 U/L) and gamma-glutamyltranspeptidase (301 U/L; reference range <52 U/L) but normal levels of aspartate and alanine aminotransferases. Abdominal echography and computed tomography revealed an abscess (6 cm x 4 cm x 4 cm) over the right lobe of the liver and a gallbladder stone. Cefoxitin (2 g every 8 hours) was given intravenously. The abscess was aspirated on the 4th day after hospitalization. Two isolates (isolates B1 and B2) of *K. pneumoniae* with different colonial morphotypes and resistotypes (as in isolates A1 and A2, respectively, from patient A) were found in one aspirate culture, and two isolates (isolates B3 and B4) of the same species with different phenotypes (as in isolates B1 and B2, respectively) were recovered from one set of blood cultures, performed at admission. Cefoxitin was discontinued, and cefotaxime (2 g every 6 hours) was administered. His fever resolved 2 days after cefotaxime administration was begun.
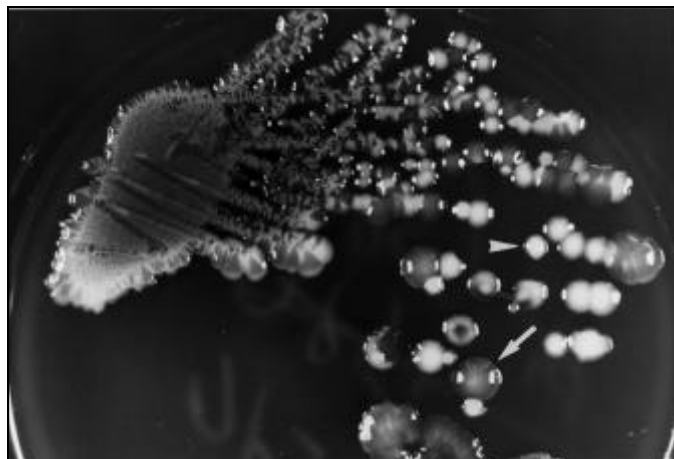
Figure 1. Colonial morphology of *Klebsiella pneumoniae* grown on a primary isolation plate (trypticase soy agar supplemented with 5% sheep blood) from the abscess aspirate of patient A after 24 hours of incubation. The arrow shows a mucoid opaque colony (isolate A1). The arrowhead shows a nonmucoid white colony (isolate A2).

*National Taiwan University Hospital, Taipei, Taiwan; †National Cheng-Kung University Medical College, Tainan, Taiwan; and ‡National Taiwan University College of Medicine, Taipei, Taiwan

The patient received cefotaxime for 15 days, followed by oral cefixime (200 mg every 12 hours) for 7 weeks. Follow-up echography showed the abscess had disappeared.

In vitro susceptibilities of 14 antimicrobial agents for these isolates were determined by using the standard agar dilution method (7). Biotyping of thee isolates was performed with the API ID32 GN system (bioMerieux, Marcy I'Etoile, France). Random amplified polymorphic DNA (RAPD) patterns of the six isolates were determined by means of arbitrarily primed polymerase chain reaction, as described in our previous report (8). A total of four primers were used: M13 (5'-TTATG-TAAAACGACGGCCAGT-3'), OPH2, OPA3, and OPA9 (Operon Technologies, Inc., Alameda, CA). Pulsotypes of these isolates were determined by pulsed-field gel electrophoresis; plasmid analysis and conjugative experiment were performed as described (3). For comparisons, molecular typing of an additional two *K. pneumoniae* isolates recovered from two patients seen at our hospital in 1999 were performed simultaneously.

The cefoxitin- and cefuroxime-resistant isolates (isolates A2, B2, and B4) showed two- to four-fold higher MICs of cefotaxime and ceftriaxone (MICs 0.5-1 µg/mL), ceftazidime (MICs 1 µg/mL), flomoxef (MICs 0.5-2 µg/mL), ticarcillin-clavulanate (MICs 8 µg/mL), piperacillin-tazobactam (MICs 4-8 µg/mL), cefepime (MICs 0.5-1 µg/mL), cefpirome (MICs 0.12-1 µg/mL), imipenem (MICs 2 µg/mL), and meropenem (MICs 0.12 µg/mL) than those of the cefoxitin-susceptible isolates (isolates A1, B1, and B3). All isolates were susceptible to the above antibiotics and aminoglycosides (gentamicin and amikacin).

As shown in the Table, the identity of biotypes, RAPD patterns with four primers, and pulsotypes (Figure 2) was clearly demonstrated for the isolates with two different phenotypes
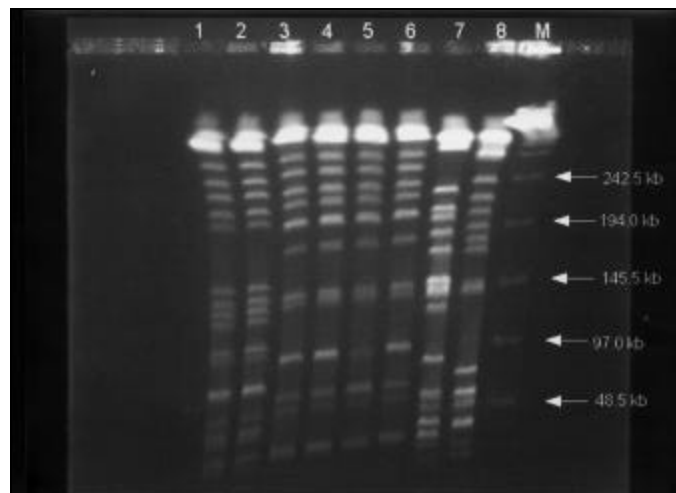

Figure 2. Pulsed-field gel electrophoresis profiles of XbaI-digested genomic DNAs from eight *Klebsiella pneumoniae* isolates. Lanes 1 and 2, profiles for isolates A1 and A2 (from patient A); lines 3 to 6, profiles for isolates B1 to B4 (from patient B), respectively; and lanes 7 and 8, isolates of *K. pneumoniae* from two other patients used as control strains. Lane M, bacteriophage lamdba DNA concatemers (GibcoBRL, Gaithersburg, MD)

from each patient, suggesting that they belonged to a single clone in each patient (clone 1, isolates A1 and A2; clone 2, isolates B1 to B4). Different clones isolated from two patients seen within 1 week indicate that no outbreak occurred. Isolates A1 and A2 both had two plasmids (50 kb and 5 kb). Only one plasmid (30 kb) was found in each of the four isolates (B1 to B4) of patient 2. All these plasmids cannot transfer to *Escherichia coli* C600.

Conclusions

Although infection caused by a single clone of one bacterial species that simultaneously possessed two obviously different

Table. Clinical characteristics of two diabetic patients with liver abscess and microbiologic characteristics of *Klebsiella pneumoniae* isolates recovered from them

| Patient designation (age, yr/ gender) | Antibiotic treatment (days) | Sources of isolate/ designation | Colonial morphotype | Resistotype (MIC, µg/mL)[a] | | | | Biotype | RAPD pattern |
				AMP	CZ	FOX (CXM)	CTX (CRO)		
A (42/M)	Ceftriaxone (10	Abscess fluid							
	Imipenem (24)	A1	Mucoid	64	4	16	0.12	I	a/1
	Ciprofloxacin (21)	A2	Nonmucoid	128	32	128	1	I	a/1
B (66/M)	Cefoxitin (10)	Abscess fluid							
	Cefotaxime (15)	B1	Mucoid	32	2	4	0.06	I	b/2
	Cefixime (35)	B2	Nonmucoid	128	32	128	0.5	I	b/2
		Blood samples							
		B3	Mucoid	32	2	4	0.03	I	b/2
		B4	Nonmucoid	128	64	256	0.5	I	b/2

[a]AMP, ampicillin; CZ, cefazolin; FOX, cefoxitin, CXM, cefuroxime, CTX, cefotaxime, CRO, ceftriaxone.
RAPD = random amplified polymorphic DNA.

colonial morphotypes has been noted previously (8), infection caused by a single clone of *K pneumoniae* exhibiting two discrete colonial morphotypes and resistotypes has never been reported. Primary liver abscess due to *K. pneumoniae* having resistance to second-generation cephalosporins (MIC up to 128 µg/mL for isolate B2 and 256 µg/mL for isolate B4 in patient B) and higher MICs of third-generation cephalosporins (MICs up to 1 µg/mL for isolate A2 in patient A) has also not been previously reported. We believe that this decreased in vitro susceptibility contributed to the unsatisfactory response to treatment with these agents.

The genetic basis for the resistance to cephalosporins and for the mucoid material synthesis by some gram-negative bacteria may be chromosomally determined or dependent on the acquisition of a specific plasmid or phage (5,9,10). The resistant and mucoid material synthesis may also be regulated by the environment in which the bacteria grow (5,9,10). The association of a specific plasmid with the presence of mucoid phenotype was not found in our isolates because both mucoid and nonmucoid strains of the two clones of *K. pneumoniae* had identical plasmid profiles. The mechanisms of the coexistence of two phenotypically distinct isolates within a single clone *K. pneumoniae* in abscess fluid, blood, or both should be investigated. The mechanism of cefoxitin resistance in our isolates was unclear because of the failure of transferability of the plasmids existing in our isolates to the susceptible recipient. Whether this resistance was chromosome mediated or caused by other mechanisms needs further investigations.

These two cases suggest that primary liver abscess caused by multiresistant strains of *K. pneumoniae* in diabetic patients may be an emerging problem in Taiwan. The belief that only strains of *K. pneumoniae* that are susceptible to cephalosporins cause primary liver abscess has now been disproved.

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

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Address for correspondence: Kwen-Tay Luh, Department of Laboratory Medicine, National Taiwan University Hospital, No. 7 Chung-Shan South Road, Taipei, Taiwan; fax: 886-2-23224263; e-mail: luhkt@ha.mc.ntu.edu.tw

The Emerging Infectious Diseases Journal: A Time of Transition

Joseph E. McDade and James M. Hughes

Joseph E. McDade   **James M. Hughes**

Change is a constant for Emerging Infectious Diseases. Since its inception in 1995, the journal has undergone many transformations: its content has broadened, its format has become more distinctive, and its frequency of publication has increased, from quarterly to bimonthly in 1999 and now to monthly with this issue. Yet, amidst all the changes, Emerging Infectious Diseases has found its niche in the ranks of scientific journals, serving the needs of professionals in infectious diseases and many related disciplines. In 7 short years, the journal has achieved a high impact factor (#3 of 36 infectious disease journals, ISI Citation Reports, 2000); indexing in Medline, Current Contents, and other major databases; translations into Spanish, Japanese, Chinese, and French; and a circulation of more than 45,000 subscribers to its print and electronic versions. The journal has also provided a new communication channel through which to encourage global investigation and dialogue on the many issues, challenges, and opportunities posed by emerging infectious diseases.

The goals of the journal stem directly from the Centers for Disease Control and Prevention's (CDC's) action plan against emerging infections: recognition of new and reemerging infections; understanding of factors involved in disease emergence, prevention, and elimination; and fast and broad dissemination of reliable information on emerging infections

around the world. Toward these goals, the journal 1) provides information on factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures; 2) reports laboratory and epidemiologic findings within a broader public health perspective; 3) provides timely updates of infectious disease trends and research (new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports); 4) publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs; 5) encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues; and 6) harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

In pursuing these goals, the journal has set a high standard of quality, not only for its peer-reviewed scientific content but also for the readability and accessibility of this content. A modern journal functions in a fast-paced world of information overload, instant access, overburdened scientific audience, and the accidental broader audience created by the World-Wide Web. To fulfill the increasing expectations of an expanding audience that includes scientists in many disciplines, public health generalists, students, and the public, articles are edited for language, communication effectiveness, style, and length. The human aspect of scientific research is always kept in the forefront by the association of scientific research with works of art on the journal's cover and by

"Another Dimension" articles, reminding the reader that, in the end, the purpose of all scientific endeavor is the betterment of humanity and the improvement of the quality of life for all people.

The journal relies on a broad international authorship base and rigorous independent peer review to provide accurate and reliable scientific information on emerging infections, free of charge, around the globe. The journal draws authors and readers from professionals in infectious diseases and many other scientific disciplines in the United States and abroad, specialists in academia, industry, clinical practice, and public health, as well as economics, demography, and sociology. To expedite dissemination of up-to-the-minute information, from its inception Emerging Infectious Diseases has published articles online ahead of print.

Since 1995, Emerging Infectious Diseases has published new scientific research, analysis and commentary, policy reviews, and concise synopses on a broad array of infectious disease topics. Coverage has included topics as diverse as *Morbillivirus* in Australia, tuberculosis trends in Japan, antimicrobial resistance in Europe, infectious disease emergence in New Zealand, genomics and bacterial pathogenesis, amphibian population declines, the role of migratory birds in the spread of *West Nile virus*, infections in the health-care setting, bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease, and bioterrorism.

In 2002, the journal remains at the crossroads of change. Upon my retirement from CDC, D. Peter Drotman, a senior scientist with the National Center for Infectious Diseases, became Interim Editor while a search is conducted for a new Editor-in-Chief. To the role of Interim Editor, Dr. Drotman brings a broad knowledge of infectious diseases, effective leadership, and boundless

enthusiasm. A veteran of the successful World Health Organization Smallpox Eradication Programme, he was among the first CDC scientists assigned to investigate cases of what was later to be named AIDS.
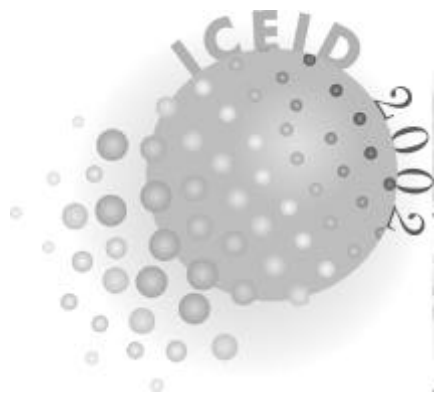
In the meantime, infections continue to emerge: new infections resulting from changes or evolution of existing organisms, known infections spreading to new geographic areas or populations, previously unrecognized infections appearing in areas undergoing ecologic transformation, old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures. The recent appearance of *West Nile virus* in the United States and the use of *Bacillus anthracis* in recent bioterrorism attacks are reminders of the need for vigilance and the undiminished potential for global dissemination of infectious agents.

As a monthly journal in 2002, Emerging Infectious Diseases will continue to track and analyze infectious disease trends and to encourage investigation and timely communication of emerging threats and critical related issues around the world. Advances in electronic publishing allow unprecedented speed in disseminating public health information and create new opportunities for innovation and improvements in communication. Electronic submission and peer review of manuscripts, online publication ahead of print, online-only publication, and convenient links to other sources of biomedical information are only the beginning.

Dr. McDade is Founding Editor, Emerging Infectious Diseases Journal. Dr. Hughes is Director, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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.

EMERGING INFECTIOUS DISEASES

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms
- Known infections spreading to new geographic areas or populations
- Previously unrecognized infections appearing in areas undergoing ecologic transformation
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and *West Nile virus* infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

- Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
- Reports laboratory and epidemiologic findings within a broader public health perspective.
- Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

- Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
- Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
- Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Randomized Controlled Trial of Active Physician-Based Surveillance of Foodborne Illness

To the Editor: In New South Wales, Australia, physicians are obliged to report to public health authorities instances of foodborne illness in two or more cases related to a common source. This reporting of cases on a clinical basis complements laboratory-based surveillance of foodborne illness and is an essential form of surveillance in situations in which clinical samples may not be collected or in which specific diagnostic testing is not routinely available. Although cases of foodborne illness are increasing, substantial underreporting to health authorities is believed likely (1,2). However, reporting of foodborne illness and investigation of identified outbreaks are important forms of health protection (1-4).

In a pilot study, we examined whether notification of single (rather than multiple) cases, active surveillance, or both would improve the reporting of foodborne illness by family physicians and thus its detection in the community.

St. George Division of General Practice, one of four networks of family physicians located in the southeastern quadrant of Sydney within the jurisdiction of the South Eastern Sydney Public Health Unit, offered to participate in the study. Passive surveillance consisted of writing to all 329 members of the St. George Division asking them to report any single case of foodborne illness on a purpose-designed form that could be faxed to the Public Health Unit. Reports remained unidentifiable unless the patient gave the physician consent for Public Health Unit follow-up. The active surveillance group comprised 34 randomly selected St. George Division members who, in addition to being sent the written information, were contacted by telephone once every 3 weeks.

Over the 12-week study period from August to November 1999, St. George Division physicians made 39 reports , 31 (79%) by facsimile and 8 by mail; in contrast, no reports of foodborne illlness were received from the other 900 family physicians practicing in southeastern Sydney. Of the 39 notifications, 26 were received from 12 (35%) of 34 active surveillance physicians and 13 from 8 (2.7%) of the remaining 295 physicians (odds ratio 19.6 [95% confidence intervals 6.6-59]).

Consent was given for the Public Health Unit's food inspectors to follow up 13 cases, 6 of which represented multiple associated cases with possible public health implications. In one family, three members had pain, altered temperature sensation, and lower limb weakness 4 to 36 hours after eating portions of flowery cod; they were diagnosed as suffering from ciguatera poisoning. This potentially serious condition is caused by consumption of heat-stable ciguatoxin concentrated in the tissues of certain types of reef fish that have ingested toxin-producing plankton. Ciguatera poisoning has wide global distribution, including the Indo-Pacific and Caribbean regions (5); it has important public health implications because of its frequency and severity, and the fact that prompt recognition and treatment can lead to a good clinical outcome (5-7)

Better ascertainment of foodborne illness is required to improve food safety in Australia, including removing suspect foods from circulation (1,3). We found that passive surveillance of single cases increased the reporting of suspected foodborne illness by family physicians, while active surveillance based on telephone contacts elicited notification of clusters of associated cases, one of which required prompt public health action. At the least, this pilot suggests vast underreporting of food poisoning and that different strategies are available to improve reporting. A large-scale study would be required to determine the feasibility, acceptability, and value to public health of this form of enhanced surveillance.

Zhong Dong,* Mark J. Ferson,*† Peter Yankos,† Valerie Delpech,*† and Richard Hurst‡

*School of Community Medicine, University of New South Wales, Kensington, New South Wales, Australia; †South Eastern Sydney Public Health Unit, Zetland, New South Wales, Australia; and ‡St George Division of General Practice, Carlton, New South Wales, Australia

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First Reported Case of Imported Hantavirus Pulmonary Syndrome in Europe

To the Editor: We report the first imported case of hantavirus pulmonary syndrome (HPS) in Europe. The patient, a 58-year-old man, traveled in Chile and Argentina from February 2 to March 2, 2001. A keen amateur botanist, he spent several days (February 17-23) on foot in Cohaique, Puerto Monte, and the surrounding rural areas collecting plant material. On February 24 and 25, he traveled by bus to Bariloche, Argentina. On March 3, shortly after he returned to France, he had a high fever (40°C to 41°C) with myalgia and headache. On March 18, he had dyspnea, which increased during the next 2 days. On March 21, he was hospitalized in the intensive care unit of the Centre Hospitalier de Compiègne, France.

On admission, the patient's fever was still high (40°C), there was severe hypoxemia with bilateral diffuse pulmonary infiltrates, a tachyarrhythmia with auricular fibrillation and gallop, and conjunctival injection. Laboratory results indicated mild renal insufficiency (urea 12.5 mmol/L; creatininemia 180 μmol/L), hepatic cytolysis (serum glutamic-oxaloacetic transaminase 236 and serum glutamic-pyruvic transaminase 72), a moderate thrombocytopenia (platelet counts 86 000/mm^3), an inflammatory syndrome (C-reactive protein 272 mg/L), and a capillary leak syndrome (hematocrit 49%; albuminemia 20 g/L). On the night after admission, an aggravation of the cardiac function with myocarditis developed; it responded quickly to symptomatic treatment. The patient's condition improved steadily on the following days with a reduction of the pulmonary manifestations, and he was discharged on April 2.

Blood samples obtained on March 21 and March 29 were tested for the presence of antibodies to hantaviruses (*Puumala*, *Hantaan*, *Sin Nombre*, [SNV] *Seoul*, and Laguna Negra) by immunoglobulin (Ig) M-capture and IgG enzyme-linked immunosorbent assay. IgM antibodies were detected for all these antigens on the first sample, but there was no increase on the second sample. A substantial increase in IgG titer for SNV and Laguna Negra antigens was observed from the first to the second sample, but not for the other antigens. The virus could not be detected either by reverse transcription-polymerase chain reaction or by inoculation into cell culture (three passages). Since the identification in 1993 of SNV as the cause of HPS (1), numerous cases of this disease have been confirmed in various regions of North and South America. The first HPS cases associated with *Andes virus* in Argentina (2) were observed in 1995. Since then, more than 500 HPS cases have been reported in six countries of South America (Argentina, Bolivia, Brazil, Chile, Paraguay, and Uruguay), with mortality rates ranging from 30% to 70%.

Hantaviruses are rodent-borne, and each is associated with a specific rodent. Sigmodontine rodents are the vectors of hantaviruses associated with HPS. Infections are most frequently transmitted by inhalation of virus-contaminated aerosols of rodent excreta, but human-to-human transmission has also been described (3).

The patient described here was probably infected in Chile and more likely in the Puerto Monte area, where HPS cases were reported in 2001. Unfortunately, virus could not be detected because the first blood sample was obtained 2 weeks after onset of fever.

Bernadette Murgue,* **Yves Domart,†** **Daniel Coudrier,* Pierre E. Rollin,‡** **Jean Paul Darchis,† Dominique** **Merrien,† and Hervé G. Zeller***

*Institut Pasteur, Paris, France; †Centre Hospitalier, Compiègne, France; and ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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Efficacy of Interferon Alpha-2b and Ribavirin against *West Nile Virus* In Vitro

To the Editor: *West Nile virus* (WNV) infected humans in the Western Hemisphere for the first time in the late summer of 1999. During 1999 and 2000, nine deaths occurred among 80 patients with meningitis or encephalitis in New York City; Westchester County, New York; New Jersey; and Connecticut (1-3). Effective antiviral agents are unknown for infections caused by WNV. Odelola (4) described 83% survival of WNV-infected mice and eradication of virus from brain when 1.5 mg. of ribavirin was administered by intraperitoneal injection after virus inoculation. Survival of controls was 25%. More recently, Jordan et al. have reported inhibition of WNV by a relatively high concentration of ribavirin (200 μM) given after infection of human oligodendroglial cells in vitro (5). Shahar et al. (6) reported protection of fetal mouse spinal cord tissues with mouse alpha and beta interferon before inoculation with WNV. We tested human recombinant interferon alpha-2b and ribavirin for activity against WNV in a primate cell system similar to that used to measure the effect of these agents on *Bovine viral diarrhea virus*, a cultivatable, closely related surrogate for *Hepatitis C virus*. Vero cells were cultured at 37° and 5% CO_2 in a 96-well microtiter plate. Approximately 13,000 cells were seeded in each well 24 hours before specific concentrations of ribavirin or interferon alpha-2b were added. Approximately 2.5 X 10^3 PFU of WNV isolated from *Culex pipiens* (7) was added 1.5-2 hours after or before the addition of interferon alpha-2b or ribavirin to Vero cells. Forty-four hours after treatment, a colorimetric proliferation assay was used to measure viable cells in each treated well according to the protocol of Promega (Madison, WI). Cells exposed to specific concentrations of antiviral compounds, but without WNV, were used as negative controls.

Interferon alpha-2b was protective and therapeutic. Interferon alpha-2b inhibited viral cytotoxicity at low dosage when applied before or after infection of cells with WNV. For example, viral protection was observed from 3,000 U/mL to 188 U/mL 2 hours before infection of cells with WNV. Interferon alpha-2b was also therapeutic when applied after cells were infected with WNV. Virus-induced cytotoxicity was inhibited by concentrations of ≥ 5.9 U/mL when added 1.5 hours after infection (Figure). The optical density 490 values in these tests were significantly different (p<0.05, using Tukey HSD multiple comparison test)
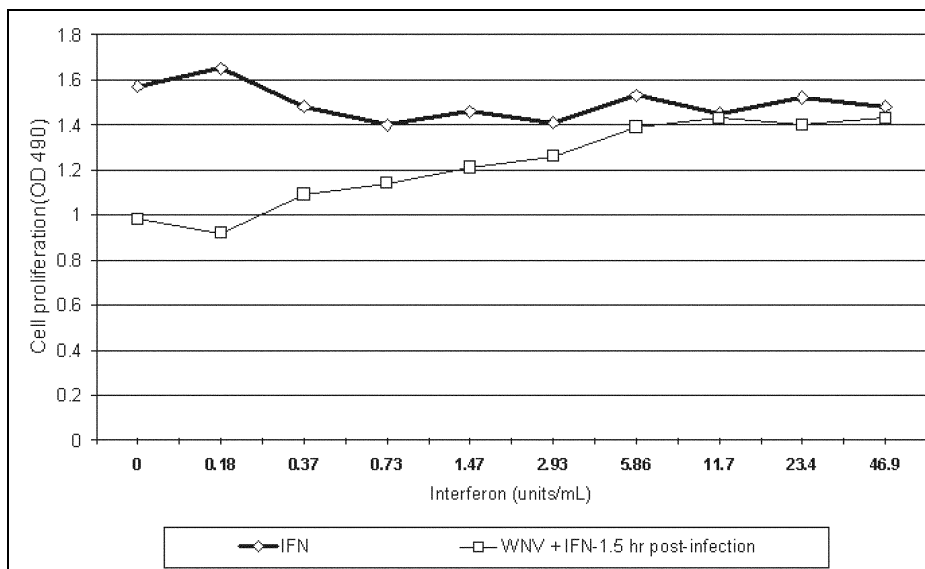
Figure. Effect of varying concentrations of interferon α-2b (FN) on *West Nile virus*-infected Vero cells. The vertical axis represents a colorimetric assay of cellular lactic dehydrogenase, which is directly proportional to cell viability and proliferation. OD = optical density.

when compared with the uninfected cells.

Ribavirin was protective but not therapeutic in vitro. Cells were protected at dosages of 400 and 500 μM but not at dosages of ≤300 μM of ribavirin applied 2 hours before infection of cells with WNV. A cytotoxic effect of ribavirin occurred at concentrations of 600-1,000 μM.

In humans, daily doses of 3 million units of interferon result in serum levels of 10-20 U/mL, well above that required for in vitro efficacy (8). In contrast, oral ribavirin doses of 2,400 mg daily yield a steady-state serum concentration of 3-4 μg/mL after several days, approximately 12-40-fold less than the in vitro inhibitory concentrations of 200-500 μM (50-125 μg/mL) noted by Jordan et al. (5) and in this study. Intravenous administration of 4 g daily, as used in the treatment of Lassa fever, would be required to reach a potentially effective serum concentration (9,10). However, intracellular accumulation and phosphorylation of ribavirin may account for its therapeutic effect in mice (4).

We conclude that interferon alpha-2b possesses greater therapeutic activity in vitro than ribavirin, with a potentially greater therapeutic ratio in humans. Whether combination therapy, as employed against hepatitis C, may be optimal requires further study.

Acknowledgments

John F. Anderson*
and James J. Rahal†

*Connecticut Agricultural Experiment Station, New Haven, Connecticut, USA; and †New York Hospital Queens and Weill College of Medicine, Cornell University, New York, New York, USA

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

Conference Summary

The Fifth International Conference on Hemorrhagic Fever with Renal Syndrome, Hantavirus Pulmonary Syndrome, and Hantaviruses

The Fifth International Conference on Hemorrhagic Fever with Renal Syndrome, Hantavirus Pulmonary Syndrome, and Hantaviruses was held June 13-16, 2001, in Annecy, a scenic resort in the French Alps. The conference, with 160 participants, was co-organized and generously hosted by the Mérieux Foundation.

Apart from Eurasia and the Americas, evidence for infections with hantaviruses now has been reported from many new areas, including the Southeast Asian countries of Cambodia, Indonesia, Taiwan, and Thailand. However, definitive and consistent evidence for the presence of these viruses still has not been reported from Africa or Australia. The occurrence of some newly recognized pathogenic Old World hantaviruses was reported (Amur virus from *Apodemus peninsulae* in the Far East and Saaremaa virus from *A. agrarius* in Europe). For the Old World, the proper taxonomy of these viruses seems to be reasonably well established. This is not the case for South American hantaviruses, presumably because rodents and their hantaviruses only recently entered the area, perhaps after the opening of the "Panama Bridge," as summarized by S. Morzunov (Reno, NV). The clinical pictures of South American hantavirus infections (D. Enria, Pergamino, Argentina) seem to be variable, intermediate between severe North American hantavirus pulmonary syndrome (HPS) and Eurasian hemorrhagic fever with renal syndrome (HFRS), and include mild cases, hemorrhagic cases, and those characterized by renal and neurologic signs.Additional evidence was presented for the occur-

rence of clustered cases, further suggesting the possibility of person-to-person transmission, in southern Argentina, Chile, and Brazil. Much additional work is required to prove such a hypothesis.

Transmission of hantaviruses in carrier rodents is complex and may be attributable to decreased biodiversity of rodent reservoir communities (J. Mills, Atlanta, GA). Although it is well known that wounds and infections are correlated, deer mice in laboratory colonies shed very little *Sin Nombre virus_* (SNV). According to S. Klein (Baltimore, MD), intact male rats shed more *Seoul virus* than do females or castrated males. As reported by B. Hjelle (Albuquerque, NM), heat shock in cell cultures and cold shock in vivo can reactivate SNV, which persists in heart, lung, and, interestingly, brown fat.

The pathogenesis of hantavirus infections is not well understood. Some progress was reported towards establishing reverse genetics for hantaviruses, as the catalytic core domain of *Hantaan virus* (HNTV) RNA polymerase has been isolated (C. Jonsson, Las Cruces NM). Also of special interest was the report by E. Mackow (Stony Brook, NY), on the selective inhibition of beta3-integrin directed endothelial cell migration by pathogenic hantaviruses. Cytotoxic T cells may also play an important role in affecting vascular permeability in HFRS and HPS (H. Van Epps and F. Ennis, Worcester, MA). Interestingly, while HFRS caused by *Puumala virus* may lead to increased blood pressure (J. Mustonen, Tampere, Finland) and HPS by SNV to pulmonary sequelae (D. Goade, Albuquerque, NM) as long-term effects, both HFRS and HPS can be characterized by increased proteinuria as a late consequence. Several reports on multiple cytokine mRNA and protein responses in cultured cells and in patients were, to say the least, somewhat contradictory; more work is needed to clarify these phenomena.

Classical Salk-type hantavirus vaccines have been widely and successfully used for a number of years in Korea and China. Meanwhile, more and more

sophisticated recombinant and DNA vaccines are being developed in Europe and North America, but none has entered the market. This work is, however, producing highly interesting results, such as the *Andes virus* lethal model in adult hamsters (J. Hooper, Frederick, MD) and the recombinant human antibodies with good therapeutic potential (J. Koch, Heidelberg, Germany).

In summary, the 3 days in Annecy provided a comprehensive progress report of the field, one that may be summarized by Mark Twain's words, "Interesting if true, interesting anyway." The participants of the conference were confident enough to found "The International Society for Hantaviruses and Hantaviral Diseases" and elected Ho Wang Lee (Seoul, Korea), the discoverer of HV, the prototype HTNV, as its first president.

Antti Vaheri, Haartman Institute, Helsinki, Finland

Charles Calisher, Colorado State University, Fort Collins, Colorado

Erratum Vol. 7 No. 6

In "The Changing Epidemiology of Malaria in Minnesota," S.A. Seys and J.B. Bender, a number was inadvertently inserted into a sentence. On page 993, column 2, second full paragraph, the third sentence should read, "Among the patients who were born abroad, sites of malarial infection were Africa and Asia."

We regret any confusion this error may have caused.

About the Cover

**The Old Port of Dubrovnik
(watercolor, 18th century) Anonymous**

Provided courtesy of Dr. Andreja Tambic-Andrana

This 18th-century painting depicts quarantine activities at the port of Dubrovnik, for many centuries one of the largest cultural centers in Croatia and a major trading center on the Dalmatian coast. Because trade with the East and the West was the driving force behind the development of Dubrovnik, suspension of trade during plague epidemics in the 14th century would have been disastrous for the city. Therefore, on July 27, 1377, the Great Council of Dubrovnik introduced in a decree a measure that would both protect against plague epidemics and free trade with eastern countries from which these epidemics usually spread. The text of this decree can be seen in Volume 78, chapter 49 of the Liber Viridis. The original document, which is kept in the Archives of Dubrovnik, states that before entering the city, newcomers had to spend 30 days in a restricted location awaiting to see whether the symptoms of plague would develop. Later on, isolation was prolonged to 40 days and was called quarantine.

The word quarantine, used to describe isolation to prevent spread of infection, comes from the Latin word "quaranta," meaning 40, because the isolation lasted for 40 days. Along with Venice and Milan, Dubrovnik was among the first cities in the world to introduce isolation as a measure to control the spread of infectious disease and the first city to have a documented organization of quarantine. Over the centuries, other epidemic diseases (leprosy, smallpox, dysentery) were recorded in the archives of Dubrovnik, and other specialized institutions (e.g., leprosaria) were organized outside the city. In 1590, quarantine activities were moved to the complex of houses near the east city gates, as can be seen on the painting (red-roofed houses at the bottom right of the painting). Isolation proved to be effective; none of the plague epidemics that occurred in centuries to come was as devastating as Black Death, which spread throughout the world in the mid-14th century. Isolation is probably the greatest achievement of medieval medicine, and the quarantine of Dubrovnik is an important development in the medical heritage of Dubrovnik and Croatia.

In the next issue of

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A Peer-Reviewed Journal Tracking and Analyzing Disease Trends   Vol.8, No.2, February 2002

Vector Interactions and Molecular Adaptations of Lyme Disease and Relapsing Fever Spirochetes Associated with Transmission by Ticks

Other Articles Include

Traditional and Molecular Techniques for the Study of Emerging Bacterial Diseases: One Laboratory's Perspective?

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For a complete list of articles included in the February issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm

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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.

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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

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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.