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# Bacterial Resistance to Penicillin G by Decreased Affinity of Penicillin-Binding Proteins: A Mathematical Model

**L. Temime,\* P.Y. Boëlle,\* P. Courvalin,† and D. Guillemot†**

*Streptococcus pneumoniae* and *Neisseria meningitidis* have very similar mechanisms of resistance to penicillin G. Although penicillin resistance is now common in *S. pneumoniae*, it is still rare in *N. meningitidis*. Using a mathematical model, we studied determinants of this difference and attempted to anticipate trends in meningococcal resistance to penicillin G. The model predicted that pneumococcal resistance in a population similar to that of France might emerge after 20 years of widespread use of β-lactam antibiotics; this period may vary from 10 to 30 years. The distribution of resistance levels became bimodal with time, a pattern that has been observed worldwide. The model suggests that simple differences in the natural history of colonization, interhuman contact, and exposure to β-lactam antibiotics explain major differences in the epidemiology of resistance of *S. pneumoniae* and *N. meningitidis*.

*Streptococcus pneumoniae* and *Neisseria meningitidis* have very similar mechanisms of resistance to penicillin G, which are mediated by the decreased affinity of penicillin-binding proteins (PBPs) (1–3). However, the epidemiology of resistance of these two bacteria exhibit very different patterns.

*S. pneumoniae* strains with decreased susceptibility have been found frequently over the last decade, and most of them now have a penicillin G MIC greater than 2 μg/mL (4,5). By contrast, for *N. meningitidis*, reports of high levels of resistance remain anecdotal, even though decreased susceptibility has become more frequent (6).

Pneumococcal resistance has already given rise to therapeutic problems (7). Because meningococcal infections are highly lethal, meningococcal resistance is a major concern. Therefore, better understanding of *S. pneumoniae* resistance selection and establishing whether meningococcal resistance could increase are important.

In both *S. pneumoniae* and *N. meningitidis*, humans are the only reservoir, and asymptomatic colonization is frequent. However, the natural history of colonization differs in these two bacterial species. The average colonization duration of *S. pneumoniae* is approximately 2 to 3 months (8), whereas duration is approximately 10 months for *N. meningitidis* (9). Asymptomatic carriage of *S. pneumoniae* peaks during the first 2 years of life and then gradually declines (10). By contrast, carriage of *N. meningitidis* peaks in young adults (9), which implies a difference in antibiotic exposure and therefore in the

selection pressure borne by these bacteria, as young children are treated more frequently than young adults.

Mathematical models can be used to explain how these factors interact in the selection of resistant strains and lead to different trends. Models of transmission have been developed to examine how antibiotic use affects the colonization rate of resistant commensal bacteria in human populations (11), to examine treatment protocols for resistance prevention (12), and to predict future trends (13). However, these models are based on a priori hypotheses which, in general, assume that the impact of antibiotic exposure does not differ according to the mechanism of resistance and do not consider the particular natural history of the colonization of the bacterial species.

We describe a mathematical model of the emergence and diffusion of bacterial resistance in the community. This model is specific to the mechanism of resistance to penicillin G common to *S. pneumoniae* and *N. meningitidis* and mediated by the decrease in affinity of their PBPs. The model also takes into account the natural histories of colonization of the two bacteria.

Using this model, we first explored the case of *S. pneumoniae* and validated our predictions by using independently obtained epidemiologic data. Next, we studied *N. meningitidis* to anticipate its trends in penicillin G–resistance selection according to antibiotic exposure.

## Materials and Methods

### Microbiologic Background and Hypotheses

β-lactam antibiotics, such as penicillin G, bind to PBPs in the bacterial cell wall. In both *S. pneumoniae* and *N. meningitidis*, the main mechanism of penicillin G resistance is mediated by the alteration of these penicillin target enzymes. The genetic events leading to reduced affinity for penicillin G are 1) point mutations that confer slight increases in resistance and 2) acquisition by transformation from other commensal species of the pharynx of intragenic sequences, which leads to the synthesis of mosaic PBPs and confers higher levels of resistance (14-16).

By convention, the decrease in susceptibility to penicillin G is defined by an MIC $\geq 0.1$ μg/mL and resistance by an MIC $\geq 2$ μg/mL (5). In the laboratory, the MIC is determined by successive dilutions and presented on a $\log_2$ scale. However, genetic events may lead to a difference of less than a $\log_2$ unity between two MICs; for example, an increase from 0.04 to 0.06 μg/mL was reported by Hakenbeck et al. (17).

*\*Institut National de la Santé et de la Recherche Médicale, Unité 444, Paris, France; and †Unité des Agents Antibactériens, Institut Pasteur, Paris, France

To take this progressive evolution into account, we theorized that each bacterial generation provided an opportunity for an increase in resistance. More precisely, we randomly selected for each new generation an increase in the bacterial MIC, defined as $d$, according to the seminormal law:

$$f_\Delta(d) = 4\sqrt{\frac{2}{\pi}}e^{-8.d^2} \ .$$

With this formulation, a high probability exists that either no genetic event occurs or that such an event will only convey a small increase in MIC, although an increase to any resistance level as a result of one genetic event remains possible. In particular, a detectable genetic event, that is, an event conveying an increase in the MIC of more than one $\log_2$ unity, will occur with a frequency of $10^{-7}$, which is consistent with previous in vitro observations [18].

Bacteria colonize human hosts in large quantities. Therefore, even though the occurrence of genetic events remains possible for each bacterium, competition makes it unlikely that a host's bacterial population will suddenly be replaced by genetically altered strains. However, in the presence of antibiotic treatment, selective killing of the susceptible bacterial population may allow replacement by a less susceptible population. We therefore assumed that genetic events leading to effective bacterial replacement were only possible in treated persons. We represented the entire bacterial population of a colonized host by one MIC.

## Model Description

To reproduce the selection and spread of resistant bacteria in the community through interhuman transmission, we divided the human population under study into several groups or "compartments" (Figure 1). Each compartment was composed of persons with the same characteristics regarding colonization and antibiotic treatment. The colonized compartments were split into several subcompartments according to MIC.

Uncolonized persons are colonized after an infectious contact with colonized persons at rate $\beta$. In the absence of antibiotic treatment, the persons are then naturally decolonized after a time $1/\lambda$, regardless of MIC. This period, called the duration of carriage, is followed by a refractory period of duration $1/\theta$, during which these persons cannot be colonized again.

With antibiotic treatment, bacterial colonization is cleared with a probability $\sigma$. In persons in whom colonization is not eliminated, bacteria with a mutation towards a higher MIC may replace the original strains.

Finally, progression from the untreated category to the treated category occurs at the start of an antimicrobial treatment, which takes place with a frequency $\alpha$, and the return to the untreated category occurs when the treatment comes to an end, after an average duration of $1/\gamma$.

## Parameters

The mean duration of carriage is reportedly 2.2 months for *S. pneumoniae* and 10 months for *N. meningitidis* [8,9].
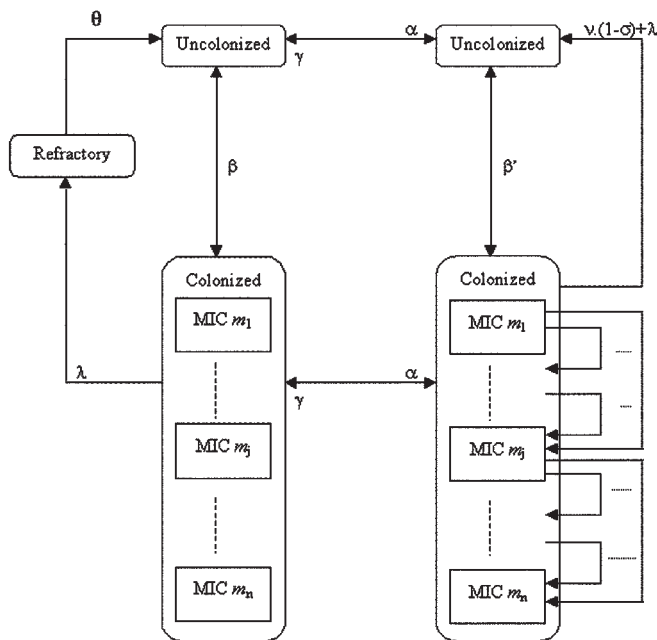


Figure 1. Structure of the model emergence and transmission of penicillin G resistance in *Streptococcus pneumoniae* and *Neisseria meningitidis*.

Although temporary systemic immunization has occurred after colonization by these bacteria [19], the exact duration of this refractory phase is not clear. We chose a duration of 2 weeks and investigated the range from 4 days to 2 months.

In studies of treatment patterns in France, the average duration of antibiotic treatment was 8 days [20,21] and the frequency of treatment changed with age. Young children may be treated several times a year with penicillin G, while healthy adults are only treated once every 4 to 5 years on average. The colonization frequency also changes with age, corresponding to colonization peaks in children for *S. pneumoniae* and in young adults for *N. meningitidis*. In our model, the population was not structured by age, but we wanted to reflect these heterogeneities. We therefore calculated effective treatment frequencies by weighting observed frequencies of treatment with probabilities of colonization according to age, which led us to study the effects of one treatment every 2 years for *S. pneumoniae* and one treatment every 3 years for *N. meningitidis*.

We assumed that, with treatment, all bacteria were submitted to the same concentration of antibiotics; therefore, we considered the probability of decolonization after treatment as a function of MIC only. A commonly used model for the effect of an antibiotic on bacteria with a given MIC according to drug concentration is the saturating model [13]. By analogy, we expressed the effect of a given antibiotic concentration in terms of the probability of nondecolonization as a function of the MIC $m$, by

$$\sigma(m) = \frac{m^3}{0.05 + m^3} \ .$$

We chose a constant infectious contact rate β in the absence of treatment. We adjusted the value of β so that the predicted proportion of carriers matched the observed values of 45% in the case of *S. pneumoniae* (10) and of 10% in the case of *N. meningitidis* (9). This gave β=0.23 weeks$^{-1}$ person$^{-1}$ for *S. pneumoniae* and β=0.026 weeks$^{-1}$ person$^{-1}$ for *N. meningitidis*. However, we assumed that a treated person had a better chance of being colonized after a contact than an untreated person if the bacteria involved had a high MIC and that colonization was less probable with susceptible bacteria. We also adopted the following sigmoidal function of the MIC *m* for the contact rate in the presence of treatment

$$\beta'(m) = \frac{(2\beta).m^3}{0.5 + m^3} \ .$$

The values of the model's parameters are specified in Table 1. Model simulations are described in the Appendix.

### *S. pneumoniae* Historical Data in France

To validate the predictions of the model for changes in resistance, we used data on *S. pneumoniae* reported to the French National Reference Center (NRC) for pneumococci (22). In short, 40–50 centers throughout France collected and sent *S. pneumoniae* strains to the NRC. Each year approximately 2,000 strains were typed and evaluated for susceptibility to various antibiotics. We used data from 1987 to 1997 and looked at changes in the distribution of penicillin G MICs over time.

## Results

### Predictions for *S. pneumoniae*

#### Emergence

By applying the model to a population in which all the pneumococci were initially susceptible to penicillin G (MIC ≤ 0.06 µg/mL), we determined the time of emergence of the first strains with decreased susceptibility (MIC = 0.125, 0.25, 0.5, and 1 µg/mL), as well as the first resistant strains (MIC = 2 µg/mL) and highly resistant strains (MIC = 4 µg/mL). The

model also provided information on the variability of these emergence times (Figure 2a). In particular, the model predicted the emergence of high resistance levels (MIC >2 µg/mL) after approximately 20 years of antibiotic use.

At low resistance levels, the mean time to emergence depended strongly on MIC. At higher levels (MIC ≥1 µg/mL), however, it reached a plateau, as the lag between the emergence of two successive levels decreased. The variability of these estimated times to emergence was marked, ranging from 10 to 30 years when starting from the same situation for the emergence of a strain with MIC 2 µg/mL.

We sought to clarify the relationship between the time required for a strain with a given MIC to be selected and the time in which the strain spreads to a large portion of the population. We determined the time at which 20% of the colonized population would be carrying strains with MIC levels ranging from >0.125 to 4 µg/mL (Figure 2a). This time appeared to depend very little on the MIC, even at low resistance levels, in contrast to the time to emergence, which began with a large increase with MIC. However, both times displayed comparable variability (data not shown).

### Transmission

We applied the model to a population in which resistance had already emerged, so that most pneumococcal strains were still susceptible to penicillin G, but some strains had high MICs. This pattern corresponds to the situation in France around 1987 (22). Figure 2b illustrates the model's predictions for the evolution with time of the distribution pneumococcal strains according to their MIC. In particular, after a few years, this distribution acquired a bimodal shape, with a peak for susceptible bacteria and another for resistant bacteria.

Figure 2c shows the evolution of pneumococcal resistance to penicillin G during 1987 to 1997, as observed by the French National Reference Center for Pneumococci (4). In 1997, the distribution of resistance levels, which was initially unimodal, exhibited a bimodal shape, with a peak for susceptible bacteria and another for resistant bacteria. This is consistent with the model's predictions (Figure 2b).

| Table 1. Model parameters and their values (8,21) | | | |
|---|---|---|---|
| Parameters (at MIC *m*) | | Pneumococci | Meningococci |
| Treatment duration | $1/\gamma$ | 8 d | 8 d |
| Weighted frequency of treatment | $\alpha$ | 1 / 2 y | 1 / 3 y |
| Refractory phase duration | $1/\theta$ | 2 wk | 2 wk |
| Carriage duration | $1/\lambda$ | 2.2 mo | 10 mo |
| Time before antibiotic action | $1/\nu$ | 4 d | 4 d |
| Contact rate (absence of treatment) | $\beta$ | 0.23 wk$^{-1}$person$^{-1}$ | 0.026 wk$^{-1}$person$^{-1}$ |
| Contact rate (presence of treatment) | $\beta'(m)$ | $\dfrac{0.46.m^3}{0.5 + m^3}$ | $\dfrac{0.052.m^3}{0.5 + m^3}$ |
| Nondecolonization probability after treatment | $\sigma(m)$ | $\dfrac{m^3}{0.05 + m^3}$ | $\dfrac{m^3}{0.05 + m^3}$ |
| MIC increase after a genetic event | $F(m)$ | Randomly selected from a seminormal law | |
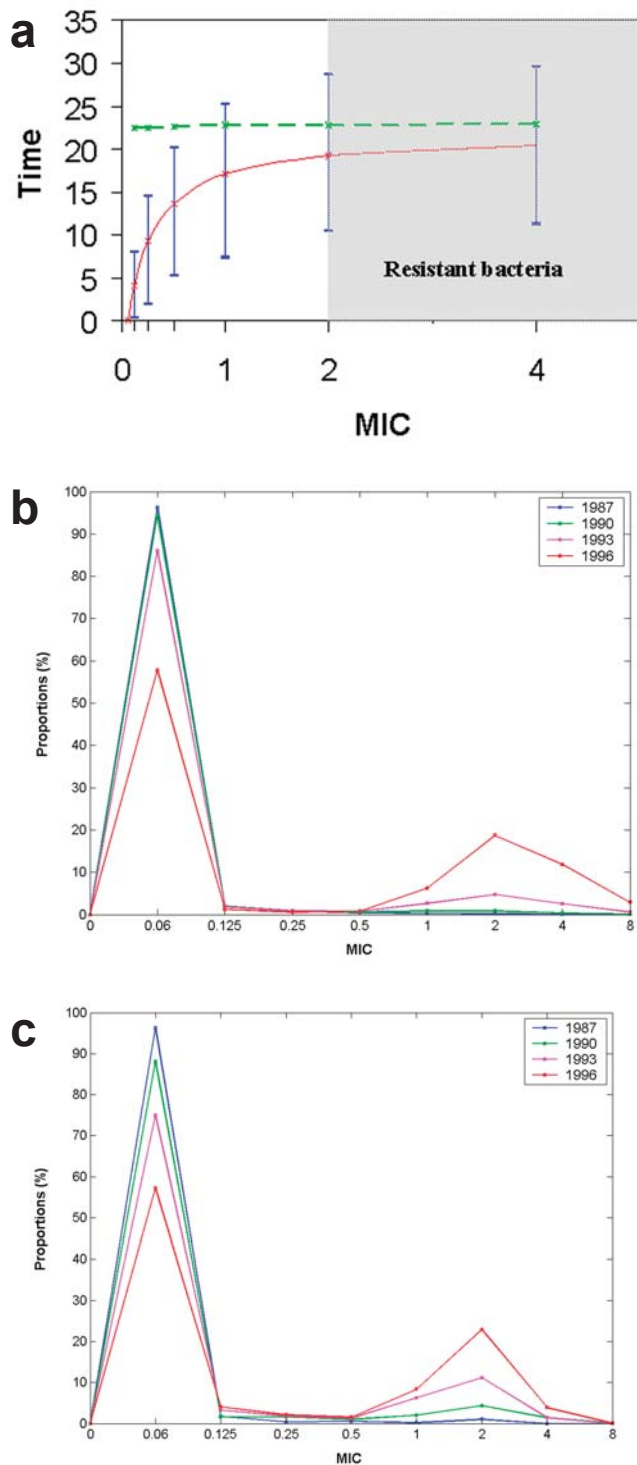
Figure 2. (a) Time to emergence of the first *Streptococcus pneumoniae* with a given MIC (full line) and time required for 20% of the bacterial population to reach this MIC (dotted line), starting from an all-susceptible pneumococcal population. Error bars correspond to stochastic variations in the model simulations (10th and 90th percentiles based on 100 simulations). (b) Simulated and (c) observed changes with time since 1987 in the distribution of resistance levels in the pneumococcal population in France. Observed data are taken from the Centre National de Référence des Pneumocoques (4).

## Predictions for *N. meningitidis*

Starting with approximately 30% of strains with reduced susceptibility to penicillin G, we used the same model to predict changes in the distribution of meningococcal MIC levels. Meningococcal resistance seemed to increase in the same way as pneumococcal resistance and also exhibited a bimodal distribution of MIC levels (Figure 3a). However, change was slower in this case because of reduced frequency of treatment in the population concerned and reduced transmissibility.

We studied a situation in which intervention would reduce the frequency of treatment by half (Figure 3b). Even under this reduced antibiotic pressure, high levels of resistance eventually appeared but with a delay of approximately 15 years.

## Discussion

In this study, we developed a mathematical model of the emergence and spread of penicillin G–resistant bacteria in the community that was specific to a resistance mechanism common to *S. pneumoniae* and *N. meningitidis*. The model shows that differences in the natural history of colonization, contact, and treatment rates can account for the differences in the epidemiology of the resistance of these two bacterial species.

Figure 2a highlights the difference between the isolation of a strain of reduced susceptibility and its spread in the community. A strain with a low resistance level does not have enough selective advantage to assure its persistence in the population. Therefore, this strain will probably disappear before a genetic event causes an increase of its MIC. For example, the large difference between the mean times of emergence of a strain with an MIC of 0.125 µg/mL and a strain with an MIC of 0.5 µg/mL corresponds to several successive processes of the emergence and elimination of strains with an MIC <0.5 µg/mL. On the contrary, at resistance levels greater than 1 µg/mL, the emergence of a strain frequently leads to its spread in the community and the prompt emergence of strains with higher resistance levels. After the first emergence of such a strain, it may take a few years to spread to 20% of the colonized population with an MIC of 2 µg/mL.

One major finding was the variability of the time to selection of bacteria with a given MIC. For example, starting from an all-susceptible bacterial population, a strain of *S. pneumoniae* with an MIC >2 µg/mL could be selected as soon as 10 years after the start of antibiotic use but also as late as 30 years later. Furthermore, this finding suggests that the absence of emergence after 30 years is unlikely, which is consistent with observations (e.g., the first penicillin G-resistant *S. pneumoniae* strains worldwide appeared around 1970, while penicillin G had been commonly used since 1950) (22).

The model predicted an increase in pneumococcal resistance leading to bimodality of MIC levels. This increase was also noted in the French data (4), as well as in other studies (23–25). Good agreement exists between the predicted and observed values (Figure 2).

A prediction of the model is that resistance of *N. meningitidis* will probably increase, although slowly, even if antibiotic

Figure 3. Simulated changes with time in the distribution of resistance levels in the meningococcal population, starting from a situation close to that of France in 2001, under (a) constant antibiotic treatment conditions (1 treatment/3 y) and (b) a frequency of treatment reduced by half (1 treatment/6 y).

Table 2. Sensitivity analysis of the model

| Parameters | | PRCC |
|---|---|---|
| Weighted frequency of treatment | $\alpha$ | 0.981651 |
| (Carriage duration)$^{-1}$ | $\lambda$ | 0.672063 |
| (Treatment duration)$^{-1}$ | $\gamma$ | -0.472343 |
| Contact rate (absence of treatment) | $\beta$ | 0.392559 |

[a]Key factors that increase (Partial Rank Correlation Coefficient [PRCC]>0) or decrease (PRCC<0) the prevalence of penicillin-resistant *Streptococcus pneumoniae* after 10 years, starting from an all-susceptible population.

ment frequencies with age, we used a treatment frequency averaged over age. Second, we only considered resistance caused by decreased affinity to PBPs, although other mechanisms may contribute to increase resistance (27,28), and we used the same mathematical description for all genetic events leading to resistance, i.e., point mutations and genetic material transfer (29) because this is supported by experimental observations (30). The very shape of the distribution used to model these increases in MIC did not alter the predictions, because selection of resistant strains in the community arose primarily by inter-human transmission. Finally, we did not include a fitness cost for resistant *S. pneumoniae* or *N. meningitidis*, although it has been found in other bacterial species (31), with the consequence that resistance progression may eventually spread faster than predicted here.

Insofar as our model takes into account both the natural history of colonization and the resistance mechanism of the bacteria considered (Appendix), the model is more realistic than general models such as those previously developed to obtain a general view of resistance (32). However, several aspects of the model could still be more complex to address specific problems, even though a certain level of simplification remains compulsory in a model. For instance, several serotypes of both *S. pneumoniae* and *N. meningitidis* cocirculate in the community. Differences exist in the transmissibility and duration of carriage of these bacteria, depending on their serotype; these differences could impact on resistance selection (9). Taking two or more bacterial serogroups into account instead of one would therefore be worth considering.

Moreover, we considered treatment with β-lactam antibiotics, whereas several antibiotics are widely used in the community. Penicillin G–resistant *S. pneumoniae* tends to be increasingly multidrug resistant (4). Taking this resistance into account may increase the impact of antibiotic exposure and therefore accelerate the changes predicted by the model. The general framework we described could be adapted to the study of other bacteria, provided the parameter values were chosen to reflect the natural history of colonization and the way in which the treatment failure probability σ (m) depends on the MIC m.

Finally, recently developed polysaccharide-protein conjugate vaccines have been shown to protect persons against symptomatic and asymptomatic colonization by *S. pneumoniae* or *N. meningitidis* (33,34). This protection is specific to the serotypes included in the vaccines. Our model could evaluate the impact of the use of such vaccines. Strategies for vaccination against *S. pneumoniae* or *N. meningitidis* may differ wide-

pressure were reduced (Figure 3b). Several parameters chosen were derived from direct measures in the community or in vitro, but others required indirect evaluations. We performed a sensitivity analysis using the Latin Hypercube sampling technique (26). This analysis showed that for predicting the percentage of resistant bacteria, the frequency of treatment was the most critical parameter (positively linked, Table 2), followed by the carriage duration (negatively linked), the treatment duration (positively linked), and the contact rate (positively linked). Also, a reasonable range of values for the duration of the refractory phase has little effect on model outcomes. Likewise, the choice of the constants and of the exponent of *m* in the probability of nondecolonization after treatment σ(m) and in the contact rate β′(m) do not have critical effects.

The following simplifications were adopted in the model. First, rather than considering explicitly the changes in treat-

ly; as vaccination for all children is recommended for *S. pneumoniae* (35), whereas targeted vaccination campaigns are more often conducted for *N. meningitidis* (36,37).

Ms. Temime is a doctoral student at Paris VI University. Her main interest is in the stochastic modeling of epidemics, especially applied to antibiotic resistance.

## Appendix

### Numerical Treatment of the Model

Classically, compartmental models are studied through a set of differential equations, which allows the computing of deterministic predictions. However, it is also possible to consider the transitions between compartments as random phenomena, to which a probability can be associated. This latter approach, called stochastic, offers the advantage of being more realistic than the classical deterministic version, as it takes into account the biological variability of the system and enables its measuring. We used both a deterministic and a stochastic version of the model in this study.

#### Deterministic Model

The equations used are as follows

$$
\begin{cases}
\frac{dU_{np}}{dt}(t) = -\alpha U_{np}(t) + \gamma V_{np}(t) - \beta U_{np}(t)\left[\int_0^\infty (U_p(c,t) + V_p(c,t))dc\right] + \theta U_{ref}(t) \\
\frac{dU_p}{dt}(m,t) = -(\alpha + \lambda)U_p(m,t) + \gamma V_p(m,t) + \beta U_{np}(t)\left[U_p(m,t) + V_p(m,t)\right] \\
\frac{dU_{ref}}{dt}(t) = \lambda \int_0^\infty U_p(c,t)dc - \theta U_{ref}(t) \\
\frac{dV_{np}}{dt}(t) = \alpha U_{np}(t) - \gamma V_{np}(t) - V_{np}(t)\int_0^\infty \beta'(c)\left[U_p(c,t) + V_p(c,t)\right]dc \\
\qquad + \lambda \int_0^\infty V_p(c,t)dc + \nu \int_0^\infty [1 - \sigma(c)]V_p(c,t)dc \\
\frac{dV_p}{dt}(m,t) = \alpha U_p(m,t) - (\gamma + \lambda + \nu(1 - \sigma(m)))V_p(m,t) + \beta'(m)V_{np}(t)(U_p(m,t) + V_p(m,t)) \\
\qquad -\nu\sigma(m)V_p(m,t) + \nu \int_0^m \sigma(c)\mathcal{F}(m-c)V_p(c,t)dc
\end{cases}
$$

where at time t, $U_{np}(t)$ is the proportion of uncolonized untreated hosts in the population, $U_{ref}(t)$ the proportion of untreated hosts in a refractory phase, $V_{np}(t)$ the proportion of uncolonized treated hosts, $U_p(m,t)$ the density of untreated hosts colonized with bacteria with MIC *m*, and $V_p(m,t)$ the density of treated hosts colonized with bacteria with MIC *m* (38).

#### Stochastic Model

Interested readers may contact the first author for technical details about the simulations that were performed by using Gillespie's Direct Method (39).

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Address for correspondence: Laura Temime, INSERM U444 - 27, rue Chaligny, 75571 Paris, Cedex 12; fax: +33 1 44 73 86 59; email: temime@u444.jussieu.fr

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# The Elderly and Waterborne *Cryptosporidium* Infection: Gastroenteritis Hospitalizations before and during the 1993 Milwaukee Outbreak

**Elena N. Naumova,\* Andrey I. Egorov,\* Robert D. Morris,\* and Jeffrey K. Griffiths\***

We used the Temporal Exposure Response Surfaces modeling technique to examine the association between gastroenteritis-related emergency room visits and hospitalizations in the elderly and drinking water turbidity before and during the 1993 Milwaukee waterborne *Cryptosporidium* outbreak. Before the outbreak, the rate of such events increased with age in the elderly (p<0.001), suggesting that the elderly are at an increased risk. During the outbreak, strong associations between turbidity and gastroenteritis-related emergency room visits and hospitalizations occurred at temporal lags of 5–6 days (consistent with the *Cryptosporidium* incubation period). A pronounced second wave of these illnesses in the elderly peaked at 13 days. This wave represented approximately 40% of all excess cases in the elderly. Our findings suggest that the elderly had an increased risk of severe disease due to Cryptosporidium infection, with a shorter incubation period than has been previously reported in all adults and with a high risk for secondary person-to-person transmission.

The elderly are a population at higher risk for infections (1). Changes in immune system and gastrointestinal functions occur with aging, leading to increased susceptibility to enteric infections (2–4). The elderly, along with children and pregnant women, are recognized by the U.S. Environmental Protection Agency as being sensitive subpopulations for waterborne diseases (5). Other researchers have recognized the elderly population as a potential sentinel group for surveillance of cryptosporidiosis (6). However, the degree of this increased sensitivity to specific gastrointestinal infections is not well characterized. In the United States, most prospective studies of enteric disease in the elderly were conducted two decades ago or earlier, when diagnostic techniques were limited, and many pathogens such as *Cryptosporidium, Cyclospora,* microsporidia, and *Escherichia coli* O157:H7 were not widely recognized and routinely diagnosed (7–9). Although diagnostic techniques have improved, a substantial proportion of gastrointestinal illness in the elderly and general population remains routinely undiagnosed. For example, a recent prospective study of gastroenteritis in sentinel general practices in the

Netherlands found that the causative agent could be detected in only 40% of all patients (10).

Contaminated drinking water is a well-documented route of transmission for *Cryptosporidium parvum* (11). Disinfecting water by using chlorination does not inactivate this parasite, making water filtration essential in protecting public water supplies. In the spring of 1993, Milwaukee had an outbreak of waterborne cryptosporidiosis associated with increased contamination of source water and a breakdown in the water filtration process at the Howard Avenue Water Treatment Plant (the south plant) (12), causing a sharp increase in finished water turbidity. More than 400,000 persons became ill, and >100 immunocompromised persons died as a result of *Cryptosporidium* infection. This epidemic was the largest outbreak of waterborne disease reported in the United States.

In our previous studies, we demonstrated that the increased rates of acute gastrointestinal illness (gastroenteritis) in Milwaukee were significantly associated with increased drinking water turbidity (13–15). During the outbreak period, the association between drinking water turbidity and physician-diagnosed gastroenteritis was the strongest at a time lag of 7 days in children and 8–9 days in adults (15). These time lags correspond to typical incubation periods for *Cryptosporidium*. While experimental animal data demonstrate that the incubation period of cryptosporidiosis is related to immune status and dose of the pathogen (16–18), little direct information for sensitive human subpopulations exists except for persons with AIDS and malnourished children (19).

Our goal was to identify the specific features of the epidemic response in the elderly during the Milwaukee outbreak of cryptosporidiosis in 1993. We sought to identify 1) the magnitude of the association between increased drinking water turbidity and increased rate of gastroenteritis; 2) the median lag between exposure and illness, corresponding to the incubation period; and 3) the magnitude of the secondary spread. We hypothesized that the Milwaukee elderly might be more susceptible to *Cryptosporidium* infection than the nonelderly population. This higher susceptibility could, in principle, be reflected by a shorter time lag for the elderly during the epidemic, a higher overall increase in the rate of gastroenteritis

\*Tufts University School of Medicine, Boston, Massachusetts, USA

associated with increased turbidity, and an association between the rate of gastroenteritis and older age. In addition, a pronounced second postexposure peak in infections could represent a higher risk for person-to-person transmission among the elderly. Since many elderly live together in nursing homes, the risk for secondary transmission may be increased as well. We used the Health Care Financing Administration (HCFA) database and novel analytical techniques to investigate the temporal, spatial, and demographic patterns in hospitalizations and emergency room visits for acute gastrointestinal illness (GIH events) among the elderly.

## Data and Methods

### Hospitalization and Emergency Room Visits

We extracted all available records of GIH events in persons who were ≥65 years of age and resided in Milwaukee County, Wisconsin, from the HCFA database for the 480-day period from January 1, 1992, through April 24, 1993. The dataset included age, zip code, type of admission, and International Classification of Disease (ICD)-9 code. We abstracted data on ICD codes 007 through 009, 558.9, and 787.0. These codes include most cases of acute gastrointestinal illness reflected in the HCFA database.

To estimate the daily rate of reported cases of acute gastroenteritis per 100,000 elderly persons, we abstracted data from the 1990 Census for five age groups (65–69, 70–74, 75–79, 80–84, and ≥85 years of age) for each zip code in Milwaukee County. To estimate the endemic and epidemic daily rate of GIH events, we divided the study time into two parts: pre-outbreak (452 days; January 1, 1992–March 27, 1993) and outbreak (28 days; March 28, 1992–April 24, 1993).

We divided Milwaukee zip codes into three categories according to the drinking water source: the north area (the Linnwood Water Treatment Plant), the south area (the Howard Avenue Water Treatment Plant), and the central area, where water from these two plants is mixed. We estimated mean daily rate of GIH events in each area before and during the outbreak and examined geographic distribution of daily rate of gastroenteritis by using the ARC/View 3.2 GIS software (ESRI, Redlands, CA).

### Association between GIH Infections and Drinking Water Turbidity

As a surrogate measure of exposure to *Cryptosporidium* oocysts, we used daily maximum effluent water turbidity (provided by the Milwaukee Water Works) at the south water treatment plant. To examine temporal associations between effluent water turbidity and gastrointestinal illness, we generated a time series of daily counts of gastroenteritis in the south and central areas, which was regressed to a time series of turbidity data. We performed the analysis by using a Generalized Additive Model (GAM) with a nonparametric "loess" smoother for the exposure variable and a set of linear autoregressive components. The number of autoregressive components was selected by

using the bias-corrected Akaike Information Criterion (13,14). To cover the range of possible incubation periods of cryptosporidiosis (19), we conducted this analysis for time lags between exposure and illness from 0 to 18 days, one lag at a time.

To test for the significance of regression slopes, reflecting the relationship between turbidity and daily rate of GIH events at the lags consistent with the incubation period for cryptosporidiosis, we repeated this analysis with the Generalized Log-linear Models (GLM). Although we expected to see the strongest association at lags of 5–9 days, we did not force the model to follow the lag structure based on a theoretical distribution of incubation periods in the population but allowed an equal probability for any lag to be influential on the outcome. Model diagnostics and significance of regression slopes for correspondent lags were tested within the GLM framework. Lags with statistically significant slope estimates for turbidity were identified.

To visualize the lagged relationship between exposure and gastrointestinal illness, we produced the temporal exposure response surface (TERS), which reflected the changes in lagged daily rates of gastroenteritis associated with changes in turbidity. Instead of plotting 18 dose-response curves (one for each lag), we assembled them in a three-dimensional surface aligned by turbidity values. The lags at which both the GLM and GAM models predicted the strongest impact of increased turbidity on the rate of GIH events after adjusting for time-varying covariates were marked on the TERS plot.

For each time lag from 0 to 18 days, we estimated the excess daily rate of gastroenteritis associated with four levels of turbidity: 0.0–0.29, 0.3–0.49, 0.5–0.99, and 1.0–2.0 Nephelometric Turbidity Units (NTU). For a given lag, the excess rate estimate reflects the difference between the predicted epidemic daily rate at a given level of turbidity and the disease-endemic daily rate during the pre-outbreak period. All analysis was performed with S-plus 4.5 statistical software (Insightful Inc., Seattle, WA).

## Results

Daily rates of GIH events per 100,000 elderly persons by age category are listed in Table 1. During and before the outbreak, the age-specific rates of GIH events exhibited similar positive trends; on average, daily rates increased by 0.44 GIH events per 100,000 persons for every 10 additional years of age (p=0.001). During the outbreak, the daily rate was substantially higher in every age category (paired t test, p=0.002) than during the pre-outbreak period.

The geographic distribution of GIH events rates for the pre-outbreak period by zip code are shown in Figure 1. This spatial distribution of rates does not suggest any consistent spatial pattern. Before the outbreak, rates of GIH events in the elderly were similar in south, central, and north areas (Table 2). During the outbreak, rates of GIH events in elderly persons increased in all three water supply areas, but the increase was much stronger in the southern and central areas than in the northern

Table 1. Daily rates of gastroenteritis-related emergency room visits and hospitalizations per 100,000 elderly persons by age category before and during the 1993 outbreak of cryptosporidiosis, Milwaukee, Wisconsin

| Age group (y) | Elderly population | Before outbreak (452 d) | | During outbreak (28 d) | |
|---|---|---|---|---|---|
| | | No. cases | Daily rate/100,000 | No. cases | Daily rate/100,000 |
| 65–69 | 39,561 | 66 | 0.37 | 15 | 1.35 |
| 70–74 | 33,019 | 108 | 0.72 | 17 | 1.84 |
| 75–79 | 26,188 | 97 | 0.82 | 12 | 1.64 |
| 80–84 | 17,584 | 93 | 1.17 | 16 | 3.25 |
| 85+ | 14,150 | 81 | 1.27 | 9 | 2.27 |
| All | 130,502 | 445 | 0.75 | 69 | 1.89 |

area (Figure 2 and Table 2). The daily rate of GIH events in the elderly residing in the southern area during the outbreak was 2.6 times higher than in the northern area.

The time-series analysis employed turbidity data from the south plant and GIH events data from the south and central areas, which were supplied completely or partially by this plant. The mean (± standard deviation) daily rates of GIH events in these areas before and during the outbreak were 0.74 (±0.94) and 2.48 (±2.07), respectively. Before the outbreak, the daily rate did not exceed 2.5 cases per day in 98% of days. However, during the outbreak, in 7 of 28 days, the daily rate was >2.5 cases per day. Before March 1993, daily turbidity



Daily rate of GIH per 100,000 elderly before the
Milwaukee cryptosporidiosis outbreak

| | 0.0 – 0.66 | | 0.63 – 0.76 | | 0.77 – 0.86 |
|---|---|---|---|---|---|

Figure 1. Geographic distributions of age-adjusted daily rate of gastroenteritis-related emergency room visits and hospitalizations per 100,000 elderly persons for the pre-outbreak period (January 1, 1992–March 27, 1993), Milwaukee, Wisconsin.

never exceeded 0.25 NTU. Time series of daily rates of GIH events in the south and central areas and daily maximum effluent turbidity for a 60-day period, including the outbreak, are shown in Figure 3.

For the pre-outbreak period, we have not found any statistically significant associations between elevated water turbidity and rates of GIH events at any time lag. During the outbreak, statistically significant associations between elevated water turbidity and rates of GIH events were detected at time lags of 5, 6, 7, and 13 days by both the GLM and GAM models. As expected, no association existed between the exposure and the outcome (GIH events) on the same day, at a zero time lag. Associations at other lags from 1 to 18 days were positive but not statistically significant (Figure 4). Based on GLM analysis, the 95% confidence interval for the relative risk associated with 1 NTU increase in turbidity at time lags of 5 and 6 days was 1.54 to 4.48.

The results of modeling of the temporal relationship between turbidity and GIH events in the elderly during the outbreak period are demonstrated by the TERS surface on Figure 4. The strongest association between increased water turbidity and increased rates of GIH events was observed at a lag of 6 days, and the second highest peak was at a lag of 13 days. This second peak is temporally consistent with secondary person-to-person transmission. The flat portion of the surface reflects the absence of any associations at low levels of turbidity (<0.5 NTU) at any lag.

The estimates of excess daily rate of GIH events in the elderly (the difference between the GAM-predicted daily rate during the outbreak and the pre-outbreak daily rate of 0.75 cases/100,000 persons) associated with four levels of turbidity at time lags from 0 to 18 days are shown in Table 3. The lags that had significant regression slopes in the GLM model are marked in this table. The maximum impact of turbidity on the rate of GIH events in the elderly was associated with turbidity values above the turbidity standard of 1 NTU. At a 6-day lag, turbidity >1 NTU was associated with four additional cases of GIH events per day per 100,000 elderly persons. At a 13-day lag, turbidity contributed 2.7 additional GIH events per day per 100,000 elderly persons.

On the basis of crude estimates of rates (Table 2), of 55 GIH events in the elderly recorded by the HCFA database during 28 days of the outbreak in south and central water supply areas, 39
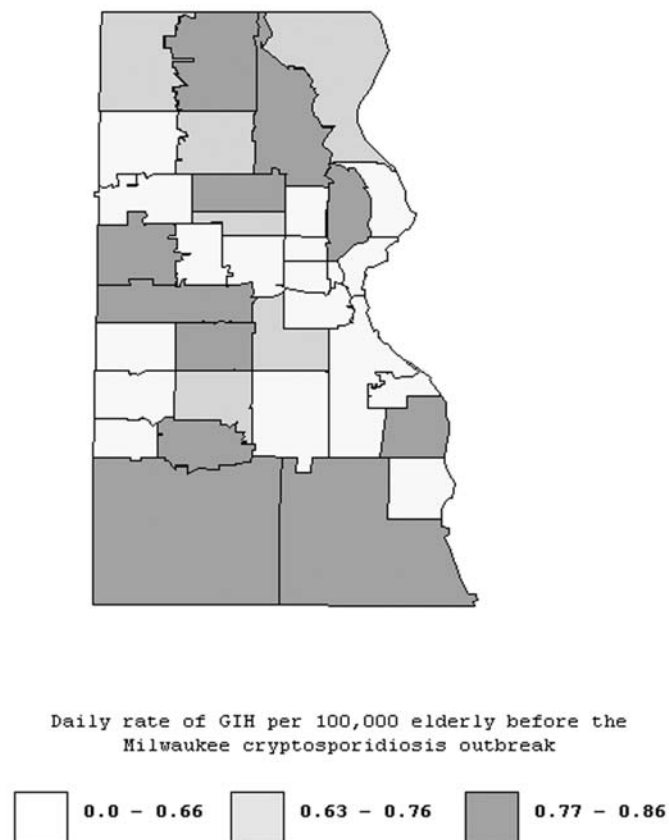
Table 2. Daily rates of gastroenteritis-related emergency room visits and hospitalizations in the elderly by water supply area before and during the 1993 outbreak of cryptosporidiosis, Milwaukee, Wisconsin

| Water supply area | No. zip codes | No. elderly | Before outbreak | | During outbreak | |
|---|---|---|---|---|---|---|
| | | | No. cases | Daily rate/100,000 | No. cases | Daily rate/100,000 |
| North | 13 | 50,747 | 182 | 0.79 | 14 | 0.99 |
| Central | 6 | 18,533 | 50 | 0.60 | 11 | 2.12 |
| South | 15 | 61,165 | 213 | 0.77 | 44 | 2.57 |

(71%) exceeded the pre-outbreak level. On the basis of the estimates of GIH excess rates in the elderly (Table 3), the total excess rate associated with turbidity >1 NTU at time lags from 0 through 18 days was 37.5 per 100,000. This rate translates into 30 excess cases of emergency room visits and hospitalizations in the 79,698 elderly in south and central Milwaukee associated with turbidity >1 NTU. Of these 30 excess cases, 18 cases (60%) occurred at time lags from 0 through 10 days postexposure (primary cases), and 12 cases (40%) occurred at time lags from 11 through 18 days postexposure (secondary cases).
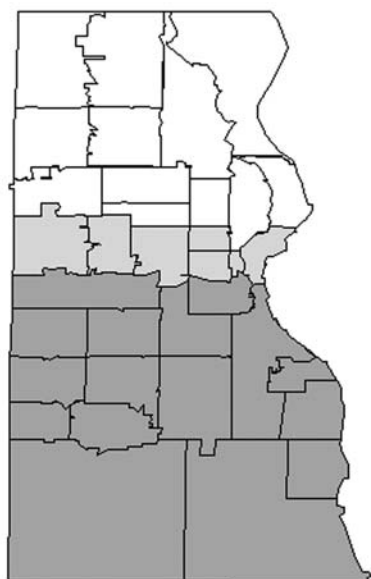


Daily age-adjusted rate (± standard deviation) of GIH per 100,000 elderly during the Milwaukee cryptosporidiosis outbreak

North plant
1.03 ± 2.7

Central area
1.34 ± 1.6

South plant
2.53 ± 2.6

Figure 2. Age-adjusted daily rates of gastroenteritis-related emergency room visits and hospitalizations per 100,000 elderly persons during the cryptosporidiosis outbreak (March 28, 1993–April 24, 1993) in three drinking water service areas (north, central, and south), Milwaukee, Wisconsin.

## Discussion

Our first finding is a positive association between age and emergency room visits and hospitalizations due to acute gastroenteritis in elderly (persons ≥65 years of age) in Milwaukee. This association was significant by age group before the outbreak (p=0.001), and significant increases were present in each category during the epidemic (p=0.002) when compared to the

pre-outbreak period. These increases are consistent with age-related susceptibility to gastrointestinal infections in the elderly. Our second finding is that statistically significant associations existed between elevated drinking water turbidity and increased rate of acute gastroenteritis in the elderly at time lags of 5, 6, 7, and 13 days during the epidemic but not in the pre-epidemic period.

In our previous study, we argued that the characteristic time lag period between a surrogate for exposure to *Cryptosporidium* oocysts, such as turbidity, and acute gastroenteritis is indicative of the incubation period for this pathogen (15). Our earlier analysis of the Milwaukee outbreak demonstrated that acute gastroenteritis cases in all adults peaked at 8–9 days postexposure to contaminated drinking water. For the purposes of comparison, we have produced the TERS plot, demonstrating the relationship between daily rates of emergency room visits and hospitalizations for gastroenteritis in all adults >17 years of age and drinking water turbidity (Figure 5) by using the datasets that we analyzed in our previous publications (13–15). In the current study, we found that the first peak in the rate in GIH events in the elderly occurred at time lags of 5–6 days (Figure 4). A comparison of these plots suggests a shorter median incubation period of cryptosporidiosis in the elderly than in all adults.

A difference in incubation periods, given the same pathogen, can be due either to a different dose of the pathogen or different host susceptibility. Experiments in genetically uni-
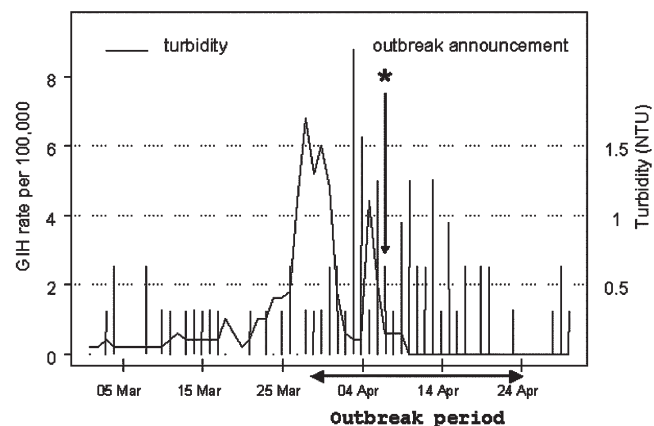


Figure 3. The fragment of the time series of daily rates of gastroenteritis-related emergency room visits and hospitalizations among Milwaukee elderly in the south and central water supply areas and daily water turbidity at the south treatment plant. The outbreak period (March 28, 1993–April 24, 1993) is indicated by blue lines; the day of announcement of the outbreak by the Milwaukee Health Department (April 7, 1993) is indicated by a green star.

Figure 4. The temporal exposure response surface plot of the lagged association between daily rate of gastroenteritis-related emergency room visits and hospitalizations in the elderly in south and central water supply areas of Milwaukee, Wisconsin (per 100,000) and water turbidity (Nephelometric Turbidity Units) at the south plant.

form γ-interferon–deficient mice have shown that the prepatent and incubation periods for *Cryptosporidium* are inversely related to the dose of parasites and can be shortened by approxi-

mately 2 days by increasing the inoculum dose by an order of magnitude (17,18). In one human volunteer study (in which the participants were not genetically uniform), an inverse (but not statistically significant) relationship was found between the dose of oocysts administered and the time to onset of infection (20). However, in the absence of any evidence that the dose of *Cryptosporidium* delivered through the public drinking water supply was substantially different (and certainly not an order of magnitude greater) for the elderly than for all adults in Milwaukee, we conclude that the observed difference in median incubation periods is probably due to a higher inherent host susceptibility to *Cryptosporidium* infection in the elderly.

The second peak in GIH rates associated with increased water turbidity occurred at 13 days postexposure. We believe that this second peak reflects the wave of secondary transmission of cryptosporidiosis from primary waterborne cases to the elderly. First, this hypothesis is consistent with an approximate doubling of the 7-day mean incubation period of cryptosporidiosis reported from this and other epidemics of cryptosporidiosis (12,15,21–25). Second, in human volunteer experiments (18,20) and a variety of other outbreaks of cryptosporidiosis (19), incubation periods of ≥13 days were very unusual.

Our third finding relates to the magnitude of the second peak in GIH rate in the elderly, the peak of presumed second-

Table 3. Generalized Additive Mode estimates of excess daily rates of gastroenteritis-related emergency room visits and hospitalizations in the elderly in south and central water supply areas during the 1993 outbreak, Milwaukee, Wisconsin[a]

| Lag (d) | 0.0–0.29 NTU[b] | 0.3–0.49 NTU | 0.5–0.99 NTU | 1.0–2.0 NTU |
|---|---|---|---|---|
| 0 | 0.57 | 0.57 | 0.56 | 0.56 |
| 1 | 0.40 | 0.50 | 0.72 | 1.20 |
| 2 | 0.27 | 0.37 | 0.58 | 1.12 |
| 3 | 0.37 | 0.46 | 0.64 | 1.10 |
| 4 | 0.33 | 0.54 | 0.94 | 1.64 |
| 5[c] | 0.31 | 0.66 | 1.38 | **3.94** |
| 6[c] | 0.22 | 0.59 | 1.33 | **4.10** |
| 7[c] | 0.15 | 0.44 | 1.02 | **3.00** |
| 8 | 0.24 | 0.61 | 1.30 | 1.97 |
| 9 | 0.29 | 0.54 | 0.97 | 1.79 |
| 10 | 0.17 | 0.54 | 1.28 | 2.53 |
| 11 | 0.18 | 0.45 | 0.95 | 1.97 |
| 12 | 0.24 | 0.46 | 0.87 | 2.01 |
| 13[c] | 0.21 | 0.58 | 1.29 | **2.68** |
| 14 | 0.09 | 0.49 | 1.23 | 2.38 |
| 15 | 0.22 | 0.48 | 0.97 | 1.80 |
| 16 | 0.37 | 0.52 | 0.76 | 1.61 |
| 17 | 0.34 | 0.50 | 0.78 | 1.05 |
| 18 | 0.38 | 0.44 | 0.52 | 1.03 |
| Average daily excess rate and standard deviation | 0.28 (0.11) | 0.51 (0.17) | 0.95 (0.28) | 1.97 (0.96) |
| Total excess rate for all lags | 5.34 | 9.73 | 18.11 | 37.48 |

[a]Per 100,000 persons at 0–18 days lag postexposure at different levels of drinking water turbidity.
[b]NTU, Nephelometric Turbidity Units.
[c]Lags in bold had statistically significant regression slopes (p <0.05).

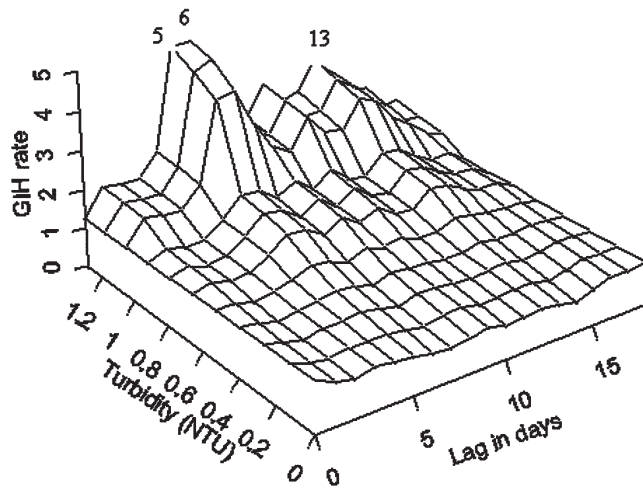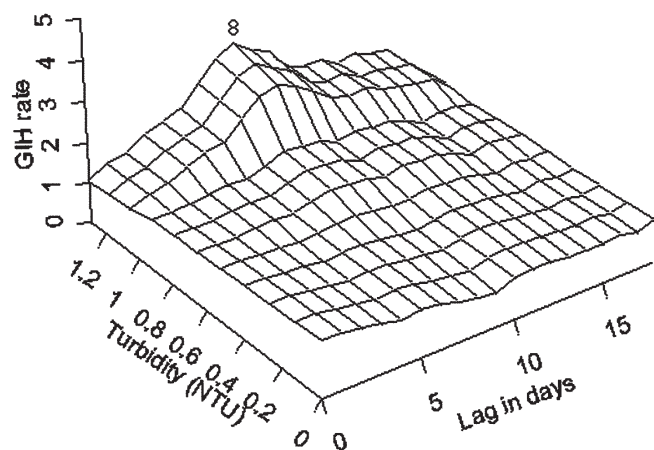Figure 5. The temporal exposure response surface plot of the lagged association between daily rate of gastroenteritis-related emergency room visits and hospitalizations in all adults in the south and central water supply area of Milwaukee, Wisconsin (per 100,000) and water turbidity (Nephelometric Turbidity Units) at the south plant.

ary transmission. Approximately 40% of excess GIH events associated with turbidity >1 NTU occurred in time lags most consistent with secondary spread. Furthermore, the relative magnitude of the increase in gastroenteritis rate at time lags consistent with secondary spread was more pronounced in the elderly than in all adults >17 years of age. (Figures 4 and 5, respectively). This finding suggests that the elderly may have a relatively higher risk for secondary person-to-person transmission. This higher risk for secondary transmission could theoretically be caused by a higher susceptibility of the elderly, a higher likelihood of exposures, especially among the elderly residing in nursing homes, or both. Relatively little is known about the overall risk for secondary transmission of *Cryptosporidium* after its introduction during an epidemic (19,26,27). MacKenzie et al. reported a secondary transmission rate in visitors to and residents of Milwaukee of 4.2% to 5%, but that study did not focus on the elderly (24). Our results in no way contradict those data.

In a retrospective microbiologic review from Rhode Island, 13 (36%) of 36 hospitalized patients identified as having had cryptosporidiosis were 63–93 years of age, with a mean of 77 years of age, and 7 of the 13 were believed to have acquired the infection nosocomially (28). Little other information about this disease in the elderly is available. A random-digit telephone survey of Milwaukee residents (12) after the 1993 outbreak identified the elderly (>70 years of age) as the subgroup with the lowest attack rate of watery diarrhea (14% vs. 26% in the general population), yet Proctor et al. (6) found that diarrhea was highly prevalent in the elderly in nursing homes during the outbreak. These apparently discrepant results may be the natural result of studying different subgroups of the elderly. In our analysis, we used age-specific data on emergency room visits and hospital admissions among all elderly as captured by the HCFA database to demonstrate that age was a risk factor among the elderly for gastroenteritis both before and during the outbreak.

While the elderly in general are more susceptible to gastrointestinal infections than other adults, gastrointestinal infections are especially prevalent in those residing in nursing homes and other similar institutions. Infectious diarrhea is the fourth most common infectious disease in the elderly residing in long-term care facilities (7). Studies have estimated the incidence of infections to be 1 to 2.59 per person-year in the elderly living in nursing homes (4,5,29) versus 0.69 per person-year in the elderly living in the community (30). Outbreaks of infectious intestinal disease are common in nursing homes and are associated with high attack rates, prolonged duration, and high disease and death rates (29,31).

Cryptosporidiosis is underdiagnosed and underreported (32,33). Cryptosporidiosis is likely to be an unrecognized cause of diarrhea in the elderly, perhaps mimicking or occurring in combination with *Clostridium difficile* (28), a well-known agent of diarrheal illness in nursing homes. Diagnostic testing for cryptosporidiosis was rarely performed before and during the Milwaukee epidemic. As most cases of cryptosporidiosis were likely to be misdiagnosed as either noninfectious gastroenteritis or masked by other pathogens, we used all ICD-9 codes that could potentially reflect cases of waterborne cryptosporidiosis.

Human volunteer studies have also established that many persons infected with *Cryptosporidium* are asymptomatic or mildly ill (20,34). Thus, most cryptosporidiosis cases were not reflected in the HCFA database in which relatively severe cases (seeking medical attention or hospitalization) were captured. While this database does not permit us to comment on these mildly ill persons, it does allow us to study the severely ill.

Among the elderly, publicity about the outbreak may have caused an increased concern and increased hospital visits. However, the peak in gastrointestinal hospitalizations among the elderly occurred on April 3, four days before the Milwaukee Health Department reported the outbreak and 5 days after the peak in water turbidity (Figure 3). Therefore, our results are unlikely to be biased by the publicity of the outbreak. In time-series analysis, in which participants serve as their own controls, the responses on a given day are compared with responses in the same population (or sample) on specified previous days. Thus, interpersonal confounding factors and biases that frequently affect the results of cross-sectional and longitudinal studies with different exposure and control groups do not affect the results of time-series analysis. However, other factors that vary in time and are correlated with both the exposure and the outcome may indeed confound the results of time-series data analysis. Our final statistical model included the most influential time-varying factor, the day of the week. In addition, the model included a set of autoregressive components to control for potential lack of temporal independence of observations. The analysis of model residuals demonstrated the adequacy of this model.

While the breakdown in the water treatment filtration process may have allowed other pathogens to enter the public drinking water supply, no concrete evidence of other pathogens

has been published. Thus, we suspect that most, if not all, of the increase in gastroenteritis detected in the elderly during this period was likely due to cryptosporidiosis. In nonoutbreak situations, cryptosporidiosis accounts for 0.5% to 5% of all cases of acute gastrointestinal illness (14,21,35). If we assume that most of the observed gastroenteritis increase in the elderly during the epidemic was due to *Cryptosporidium* infection, then the estimated magnitude of the increase in severe cryptosporidiosis cases in the elderly that resulted in hospitalization or emergency room visits is 30- to 300-fold.

The incidence of waterborne disease has been shown to be associated with the type of water supply; it is higher in communities with unfiltered surface water or mixed unfiltered surface and ground water supplies (36,37). Drinking water contamination with pathogens, such as *Giardia* and *Cryptosporidium*, has been shown to correlate with drinking water turbidity (38–40). In recognition of the importance of turbidity as an indicator of microbiologic safety of drinking water, the U.S. Environmental Protection Agency has recently released more stringent regulations to control drinking water turbidity (see Federal Register, 66:3770 and 67:1812, the Interim Enhanced Surface Water Treatment Rule and the Long Term 1 Enhanced Surface Water Treatment Rule, respectively) (41,42). Our analysis reflects the use of finished drinking water turbidity as a surrogate variable reflecting exposure to *Cryptosporidium* oocysts in water. The data on actual concentration of *Cryptosporidium* oocysts in tap water before and during the Milwaukee outbreak are not available because no prospective *Cryptosporidium* monitoring was conducted at that time.

The analytical tools we have developed in this and our previous studies allowed us to estimate the total number of attributable cases for primary waterborne exposures and for secondary transmission. In this study, we expanded our previously developed methods by estimating the excess cases associated with increased water turbidity. Standard epidemiologic investigations usually require a history of exposure to a known primary case of disease in order to link a secondary case to the outbreak. This requirement may result in an underestimation of secondary transmission, since the persons involved in this chain of transmission may not recall or recognize their contacts. Thus, the novel statistical technique that we applied for this analysis may have broad applicability to estimating the impact of secondary infections during outbreaks.

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Dr. Naumova is a biostatistician interested in the analysis of complex biological systems and the influence of intermediate events on spatial and temporal patterns. Her particular research emphasis is the development of analytical tools for time series and longitudinal data analysis applied to disease surveillance, exposure assessment, and studies of growth. Her research activities span a broad range of research programs in infectious disease and environmental epidemiology, molecular biology and immunogenetics, nutrition, and growth.

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Address for correspondence: Elena N. Naumova, Department of Family Medicine and Community Health, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA; fax: 617 636 4017; email: elena.naumova@tufts.edu

# Cost of Illness in the 1993 Waterborne *Cryptosporidium* Outbreak, Milwaukee, Wisconsin

**Phaedra S. Corso,\* Michael H. Kramer,\* Kathleen A. Blair,† David G. Addiss,\***
**Jeffrey P. Davis,‡ and Anne C. Haddix§**

To assess the total medical costs and productivity losses associated with the 1993 waterborne outbreak of cryptosporidiosis in Milwaukee, Wisconsin, including the average cost per person with mild, moderate, and severe illness, we conducted a retrospective cost-of-illness analysis using data from 11 hospitals in the greater Milwaukee area and epidemiologic data collected during the outbreak. The total cost of outbreak-associated illness was $96.2 million: $31.7 million in medical costs and $64.6 million in productivity losses. The average total costs for persons with mild, moderate, and severe illness were $116, $475, and $7,808, respectively. The potentially high cost of waterborne disease outbreaks should be considered in economic decisions regarding the safety of public drinking water supplies.

*C*ryptosporidium parvum, a protozoan parasite that causes gastrointestinal illness, is transmitted by ingestion of oocysts excreted in human or animal feces. Typical modes of transmission include person to person, animal to person, by exposure to contaminated surfaces, and by ingestion of impure food or water (1). From 1990 to 2000, at least 10 cryptosporidiosis outbreaks associated with contaminated drinking water were reported in the United States (2–5). Although the health impact of an outbreak of cryptosporidiosis originating from a contaminated public water source has been carefully documented (6), little effort has been made to estimate the economic impact of such an outbreak. This study estimates the cost of illness associated with perhaps the largest outbreak associated with a contaminated public water source ever reported in the United States. In 1993, an estimated 403,000 residents of the greater Milwaukee, Wisconsin, area (population, approximately 1.61 million) became ill when an ineffective filtration process led to the inadequate removal of *Cryptosporidium* oocysts in one of two municipal water treatment plants (6). We assessed direct medical costs and productivity losses from diarrheal illness during the Milwaukee outbreak to estimate the total cost of illness and the average cost per person with mild, moderate, and severe illness.

This cost-of-illness analysis was based on epidemiologic data collected during and after the 1993 cryptosporidiosis outbreak in Milwaukee, Wisconsin. Primary data on utilization and cost of inpatient admissions were obtained from a review of medical and financial records from hospitals in the greater Milwaukee area.

## Methods

### Epidemiologic Burden of Illness

A telephone survey of 613 households provided estimates on the total number of persons in Milwaukee experiencing mild, moderate, or severe illness as a result of the cryptosporidiosis outbreak (6). Cases were defined as residents of Milwaukee County or the surrounding four counties (Washington, Ozaukee, Racine, and Waukesha) with onset of watery diarrhea from March 1 to April 28, 1993 (the outbreak period). When disease case estimates were adjusted for normal background diarrheal disease rates, investigators estimated that 403,000 residents of the five-county area experienced illness caused by the cryptosporidiosis outbreak (6). Of this group, an estimated 354,600 persons (~88%) did not seek medical attention; 44,000 persons (~11%) were seen as outpatients; and 4,400 persons (~1%) were hospitalized.

### Cost of Illness

Following the design of the epidemiologic studies of the same outbreak, we categorized illness as mild, moderate, or severe by type of medical care sought during the outbreak period and the following 2 months (4-month study period) when persons were still likely to seek medical care (6–8). Persons with mild illness did not seek physician or emergency department care for their illness. Persons with moderate illness had at least one physician or emergency department visit but were not hospitalized. Persons with severe illness were hospitalized at least once during this period.

Previous studies and evidence collected during the outbreak suggest that underlying medical conditions such as AIDS can increase the severity of illness in persons infected with *Cryptosporidium* (9,10). To capture the effect of underlying condition on cost of illness, we further classified patients with moderate and severe illness as having no underlying condition,

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †City of Milwaukee Health Department, Milwaukee, Wisconsin, USA; ‡Wisconsin Division of Public Health, Madison, Wisconsin, USA; and §Emory University, Atlanta, Georgia, USA

an underlying condition likely treated with immunosuppressive drugs, or AIDS.

Data on utilization and average cost of inpatient services, emergency department visits, ambulance transports, and medication for persons with moderate and severe illness were obtained from a review of medical and financial records from 11 of the 14 hospitals in the greater Milwaukee area. The three nonparticipating hospitals did not differ in the number of confirmed cases of persons infected with *Cryptosporidium,* nor did they serve specialty populations that would result in higher medical costs per case. Total cost of illness was estimated from average cost of illness multiplied by the burden of illness. All clinical and financial data were recorded on standardized forms and entered into a computerized database. We did not collect information that identified patients by name or billing account number. Additional data on use of services and costs for persons with mild illness and data on productivity losses were obtained from the City of Milwaukee Health Department and published epidemiologic studies on the outbreak (6–8).

Cost-of-illness estimates for mild, moderate, and severe illness included both direct medical costs and indirect costs associated with lost productivity. Medical costs included costs for inpatient and outpatient health services, ambulance transport, and medication. Productivity losses included time lost by infected persons due to illness and the time lost by caregivers or family members to tend to ill persons. All costs are presented in 1993 U.S. dollars. We did not include litigation costs, the cost of preventive measures (e.g., switching to bottled water), intangible costs associated with pain and suffering, or the cost to the local, state, and federal government to investigate and control the outbreak.

## Medical Costs

We used several parameters to estimate the direct medical costs associated with diarrheal illness during the Milwaukee cryptosporidiosis outbreak (Table 1).

### Inpatient and Emergency Department Health Care Costs

To assess the usage and average cost of inpatient admissions (e.g., hospitalizations) and outpatient services associated with emergency department visits, we reviewed all hospital medical charts for persons with laboratory-confirmed cryptosporidiosis

Table 1. Parameter estimates for calculating the cost of diarrheal illness during the cryptosporidiosis outbreak, Milwaukee, Wisconsin, 1993[a]

| Parameter | Mild illness | Moderate illness | Severe illness |
|---|---|---|---|
| % of infected persons with diarrhea | 87.99 (6) | 10.92 (6) | 1.09 (6) |
| **Medical costs** | | | |
| Inpatient healthcare costs | | | |
| % with hospitalization | – | – | 100 |
| Cost of hospitalization | – | – | $6,312[b] |
| Outpatient healthcare costs | | | |
| % with physician visit | – | 95[c] | 29[b] |
| Cost of physician visit | – | $45[d] | $45[d] |
| % with emergency department visit | – | 5[b] | – |
| Cost of emergency department visit | – | $224[b] | – |
| Ambulance transport | | | |
| % of emergency department visit/hospitalization with ambulance transport | – | 4.9[b] | 16.3[b] |
| Cost of ambulance transport | – | $228 (11)[c] | $228 (11)[c] |
| Medication | | | |
| Self-medication before seeking healthcare | | | |
| Duration of illness before seeking health care, mean days | 4.7[e] | 5.8[b] | 18.4[b] |
| % taking medication | 30[c] | 30[b] | 29[b] |
| Cost of medication | $5.73 (12)[c] | $5.92 (12)[b] | $6.74 (12)[b] |
| Medication prescribed after seeking healthcare | | | |
| % taking medication | – | 54[b] | 48[b] |
| Cost of medication | – | $8.91 (12)[b] | $70.52 (12)[b] |
| Recurrent episodes | | | |
| % with recurrent episodes (and assuming 100% take medication) | 21 (7) | 21 (7) | 21 (7) |
| Duration of recurrent episodes, mean d | 2 (8) | 2 (8) | 2 (8) |
| Cost of medication | $2.44 (12)[c] | $2.44 (12)[c] | $2.44 (12)[c] |
| **Productivity losses** | | | |
| Productivity losses for ill persons, mean d | 1.3[e] | 3.8[e] | 13.5[e] |
| Productivity losses for caregivers, mean d | 0.1[e] | 1.3[e] | 3.9[c] |
| Earnings lost/d | $81 (13) | $81 (13) | $81 (13) |

[a]Reference nos. are in parentheses.
[b]Medical chart review.
[c]Author assumption.
[d]City of Milwaukee Health Department.
[e]Random digit dial survey conducted by the City of Milwaukee Health Department.

as identified by the hospitals' laboratory records. Because the sensitivity of diagnostic testing is relatively low, and many persons were not tested during the outbreak, we also reviewed a sample of charts for persons admitted to the emergency department or hospital with diarrhea for at least 2 days, as identified by the following diagnostic codes from the International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) listed in one of the first four diagnosis categories on the hospital discharge record: 007.20, 008.80, 009 and subcategories, 079.90, 234.10, 276 and subcategories, 558.90, and 999 and subcategories. During these admissions, either no laboratory testing was performed or tests were negative for *Cryptosporidium* and other intestinal pathogens. For the two samples, we included all costs for the inpatient admission or emergency department visit, regardless of whether the cost was directly attributable to cryptosporidiosis. Charts were excluded when the hospital admission was primarily for another condition (i.e., ICD-9-CM codes were not listed in one of the first four diagnostic categories) and when the onset of gastrointestinal illness occurred after hospital admission.

From the medical records, we collected data on resource use during hospitalization or emergency department visit, the use of ambulance transportation, self-reported use of medication before an emergency department visit or inpatient admission, and physician-prescribed medication following an emergency department visit or inpatient admission. Charges for hospitalization included diagnostic, laboratory, hospital room, and technical services (e.g., physical therapy, occupational therapy, and respiratory services); attending physician and nursing staff; medication; emergency department services; and other supplies or services not identified in the previous categories (e.g., medical-surgical supplies, clinic services).

Charges for emergency department visits and hospitalizations were converted to costs by using an average cost-to-charge ratio of 0.67, based on ratios obtained from 6 of the 11 hospitals sampled in the greater Milwaukee area; this figure is comparable to Wisconsin's average operating cost-to-charge ratio (0.70) reported for urban hospitals in 1993 (14). Charges for specialty consultations not included in the hospital bill were excluded from this analysis because insufficient data were available on the number, duration, or charges for these services.

### Outpatient and Ambulance Costs

We assumed that 95% of persons with moderate illness sought the care of a physician (one visit only) and that the remaining 5% required an emergency department visit. (Data collected from the epidemiologic investigation provided information on whether ill persons sought healthcare for their illness and whether they were hospitalized. No information was collected on whether a nonhospitalized healthcare visit was to seek physician or emergency department services. Therefore, in the absence of reliable data, we assumed that 5% of persons with moderate cryptosporidiosis went to the emergency department.) For the latter group, we assumed that no additional

physician visits were needed before or after the emergency department visit. The proportion of persons with severe illness who had a physician visit before hospitalization was obtained from chart review. We assumed that one physician visit was needed before (and none after) the hospitalization. The cost of a physician visit ($45) was obtained from data collected by the City of Milwaukee Health Department. (Since costs, and not charges, for physician visits were provided, obtaining cost-to-charge ratios was not necessary.) This figure is in the range found by other studies that have estimated the cost of a physician visit as ranging from $40 (1992 dollars) to $53 (1994 U.S. dollars) during this period (15,16).

Use of ambulance transport was indicated on the medical charts for emergency department visits and hospitalizations. Ambulance transport was used by 4.9% of those with moderate illness involving an emergency department visit compared with 16.3% of those with serious illness. We used the 1993 rate set by the City of Milwaukee ($185.50 for conveyance, $12 for minor services, and $6 per mile) for the cost of an ambulance transport, and we assumed that the average distance per transport was 5 miles (11).

### Medication Costs

For mild illness, data regarding the duration of illness were collected by the City of Milwaukee Health Department by using a random digit dial survey. Methods for this data collection were published (6). For moderate and severe illness, data regarding the duration of illness before an emergency department visit or hospitalization, the percentage of persons self-medicating during this period, and costs for medication were obtained from the medical records. We assumed the percentage of persons who self-medicated, as obtained from emergency department records for a person with moderate illness, also applied to persons with mild illness and to persons who did not use the emergency department but sought other medical care.

We estimated that all persons with mild illness who self-medicated used four 2-mg tablets of loperamide antidiarrheal medication per day or two 32-oz packs of oral rehydration solution per week, at a cost of $2.44/d. In the absence of reliable data on the duration of self-medication for a person with mild illness, we assumed that persons took medication for 50% of the duration of illness.

From the medical records, we collected detailed drug information (i.e., type, quantity, and duration) for medications prescribed upon discharge for persons with moderate and severe illness. We assumed that medications prescribed for persons with moderate illness seeking an emergency department visit also applied to persons with moderate illness seeking physician care. Retail drug prices in 1993 were used to calculate all costs (12).

Data about recurrent illness for mild, moderate, and severe illness were obtained from two investigations conducted during the outbreak (7,8). On the basis of these data, we estimated that 21% of ill persons experienced a recurrent episode of diarrhea for 2 days. As we did for persons with mild illness, we assumed

that persons with recurrent illness took medication for 50% of the duration of illness at a cost of $2.44/d.

## Productivity Losses

Productivity losses for ill persons and their caregivers were estimated from data on days lost because of illness collected by the random digit dial survey conducted by the City of Milwaukee Health Department (6). In the absence of reliable data on the days lost by caregivers of persons with severe illness, we assumed that a caregiver was needed for 50% of the number of days hospitalized. The value of missed work time by a caregiver or person with diarrheal illness was estimated by using the average annual wages for residents of Wisconsin in 1993 (13), increased by 25% to include fringe benefits. Because the type of day lost (i.e., work or leisure) was not specified in the secondary data available, we used an average daily value of $81 (annual wage plus fringe benefits, divided by 365 days) (17). We valued the time of all persons based on the productivity of the average worker, regardless of the work force status of any person.

## Results

We reviewed approximately 2,000 medical records from October 30 through November 11, 1995, and identified 378 persons who met our case definition for a moderate or severe case of cryptosporidiosis. We collected data on 155 persons who met our case definition for a moderate illness (i.e., emergency department visit only) and 223 persons who met our case definition for severe illness (i.e., a hospitalization). Seventeen percent of persons with moderate illness and 63% of persons with severe illness in our sample had laboratory-confirmed cryptosporidiosis.

Average costs of illness for persons with mild, moderate, and severe illness were $116 for mild, $475 for moderate, and $7,808 for severe (Table 2). Direct medical costs represented 2% of the average cost for persons with mild illness, 13% of the average cost for persons with moderate illness, and 82% of the average cost for persons with severe illness. The average cost of illness for all persons who experienced diarrheal illness, weighted by the proportion in each illness category, was $239 per person: $79 in medical costs and $160 in productivity losses.

The total cost of illness associated with the cryptosporidiosis outbreak in Milwaukee was approximately $96.2 million: $31.7 million in direct medical costs and $64.6 million in productivity losses (Table 3). Medical costs accounted for 33% of the total cost of illness, including $790,760 for mild illness, $2.7 million for moderate illness, and $28.2 million for severe illness. Productivity losses accounted for 67% of the total cost of illness, including $40.2 million for mild illness, $18.2 million for moderate illness, and $6.2 million for severe illness. Nearly 43% of all costs were attributable to persons with mild illness, 22% to persons with moderate illness, and 36% to persons with severe illness.

Table 2. Average cost per person with mild, moderate, and severe illness[a,b]

| Illness severity | Medical costs ($) | Productivity losses ($) | Total ($) |
|---|---|---|---|
| Mild | 2 | 113 | 116 |
| Moderate | 62 | 413 | 475 |
| Severe | 6,399 | 1,409 | 7,808 |
| Average cost of illness | 79 | 160 | 239 |

[a]Costs in 1993 United States dollars.
[b]Costs may not add up due to rounding.

## Costs for Emergency Department Visits and Hospitalizations by Underlying Condition

The average cost for an emergency department visit for persons with moderate illness was $224 (Table 4). For persons with no underlying condition (84% of emergency department visits), the average cost for an emergency department visit was $213. For persons with underlying conditions (only one patient had AIDS), the average cost for an emergency department visit was $265. The average cost for a hospitalization for persons with severe illness was $6,312, with an average length of stay of 8 days. For persons with no underlying condition (34% of hospitalizations sampled), the average cost for a hospitalization was $3,131, with an average length of stay of 5 days. For persons with an underlying condition other than AIDS (52% of hospitalizations that met the case definition), the average cost for a hospitalization was $5,520, with an average length of stay of 7 days. Persons with AIDS (14% of hospitalizations) incurred the greatest average cost of hospitalization, $17,388, with an average length of stay of 16 days.

## Discussion

The massive waterborne outbreak of cryptosporidiosis in 1993 in Milwaukee caused illness in approximately 403,000 persons and generated substantial healthcare costs and productivity losses. We estimate that on average, ill persons incurred approximately $79 in medical costs and $160 in productivity losses, resulting in $31.7 million in total medical costs and $64.6 million in total lost productivity. Since epidemiologic estimates of incidence contribute substantially to total cost estimates for any outbreak, information on average cost of illness by severity can be applied to any range of epidemiologic estimates to assess the sensitivity of total costs. For example, in the Milwaukee outbreak, the 95% confidence interval for burden (epidemiologic) of illness ranged from 370,000 to 435,000 per-

Table 3. Total cost of illness during the 1993 cryptosporidiosis outbreak[a,b]

| Illness severity | Medical costs ($) | Productivity losses ($) | Total ($) |
|---|---|---|---|
| Mild (n=354,600) | 790,760 | 40,212,000 | 41,002,000 |
| Moderate (n=44,000) | 2,710,800 | 18,176,000 | 20,887,000 |
| Severe (n=4,400) | 28,153,000 | 6,201,400 | 34,355,000 |
| Total cost of illness (n=403,000) | 31,655,000 | 64,589,000 | 96,244,000 |

[a]Costs in 1993 United States dollars.
[b]Costs may not add up due to rounding

Table 4. Average cost for emergency department visits and hospitalizations, by underlying condition[a]

| | Emergency department visit ($) | Hospitalization ($) | Average length of stay(d) |
|---|---|---|---|
| No underlying condition | 213 | 3,131 | 5 |
| Underlying condition, other than AIDS | 265 | 5,520 | 7 |
| AIDS | NA | 17,388 | 16 |
| All conditions | 224 | 6,312 | 8 |

[a]Costs in 1993 United States dollars.

sons (2,400 to 6,400 for severe cases, and 38,000 to 50,000 for moderate cases) (6). Applying these epidemiologic burden of illness estimates to the average cost per case by severity, total medical costs and productivity losses for the Milwaukee outbreak ranged from $75 to $118 million.

Although only 1% of persons who experienced diarrheal illness associated with the outbreak were hospitalized, their medical costs accounted for 89% of the total outbreak-related medical costs. Persons with suppressed immune systems were the most severely affected, accounting for 66% of hospitalizations and 74% of the total outbreak-related direct medical costs. Persons with AIDS incurred hospital costs five times greater than persons with no underlying condition. Persons with underlying conditions other than AIDS incurred almost twice the cost of hospitalization compared with persons with no underlying condition.

During the 4-month period during and after the outbreak, the productivity of Milwaukee residents and visitors who experienced diarrheal illness and their caregivers was severely affected. Although mild illness did not represent a great strain on the use of medical care resources, productivity losses were substantial given the number of persons who experienced mild illness that debilitated them in some capacity. Productivity losses accounted for 98% of total costs for persons with mild illness and 87% of total costs for persons with moderate illness.

The cost-of-illness estimates in this study are conservative for several reasons. Primary data collection from medical and financial records limited our ability to assess all costs associated with the outbreak. For example, medical and financial records lacked details about physician visits, ambulance transports, or self-medication before admission, and cost information for professional services provided during hospitalization that were billed separately. Further, an estimate of the magnitude of the occurrence of illness among visitors to the greater Milwaukee area was not made. Conservative estimates were used for any assumptions made when reliable data were not available. Second, we excluded productivity losses associated with chronic illness that might have extended beyond our 4-month study period, and we also excluded productivity losses associated with premature death. An estimated 69 deaths occurring principally among persons with AIDS were attributed to Milwaukee's cryptosporidiosis outbreak (18). Excluding the productivity losses associated with premature mortality potentially underestimates our results for total productivity losses associated with the outbreak.

While this study focused on the direct medical costs and productivity losses for illness associated with the outbreak, a broader perspective for the analysis would have included other nonmedical costs for infected persons, costs to businesses, and the cost to government agencies of controlling the outbreak and improving the public water system. Costs to government agencies alone, including the Centers for Disease Control and Prevention (CDC), the Environmental Protection Agency, the Wisconsin Division of Health (currently the Wisconsin Division of Public Health), the City of Milwaukee Health Department, the Milwaukee Water Works, and 17 local health departments, were estimated at >$2 million immediately following the outbreak (CDC, unpublished data). A class action suit filed by the residents of Milwaukee against the city continued to generate costs for the local government well beyond the immediate outbreak period. Businesses similarly experienced financial hardship during the outbreak because of employee illness, the necessity of using bottled water during the city's boil-water advisory, and a decrease in beverage and food sales overall. Unaccounted costs for the infected person include costs incurred for self-protection (i.e., the purchase of bottled water) and the pain and suffering associated with illness.

Although the $96.2 million in illness costs attributed to the Milwaukee outbreak is substantial, estimated monetized annual costs of waterborne disease in the United States have been estimated at $21.9 billion (1991 dollars) (19). This figure is based on estimates of 7.1 million cases of mild to moderate waterborne disease and 560,000 cases of severe disease (20), and an average cost per case of $2,860, including medical costs and productivity losses (21). (Average cost per case, $2,860, is based on a study of a giardiasis outbreak in Pennsylvania in 1983–1984 [21]. Although the case-fatality ratio was lower than in Milwaukee, the cost per case was higher than our estimates because of a longer duration of illness. The authors [19] note that $2,860 likely overestimates the cost of a mild case and underestimates the cost of a severe case.)

In an era of limited health resources, decision makers must choose how to allocate resources to improve the public's health. Measures taken to reduce the risk of waterborne cryptosporidiosis will also prevent other waterborne diseases. Water authorities often face the predicament of dealing with decreasing raw water quality, the high costs of new technologies, water filtration systems that do not completely remove all potentially pathogenic organisms, and increased public demand for safe water. The cost of this outbreak, which can be balanced against the cost of measures for preventing future outbreaks,[1] is a

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1In 1998, the City of Milwaukee completed an $89 million renovation of two municipal water treatment plants, which together serve approximately 800,000 people, as part of an effort to control future outbreaks of *Cryptosporidium* (22).

reminder that failure to maintain safe drinking water supplies has substantial impact on the health and economy of a community.

Dr. Corso is a health economist in the National Center for Injury Prevention and Control at the Centers for Disease Control and Prevention (CDC). During the time that this study was conducted, she was an economic analyst in the Prevention Effectiveness Branch of CDC's Epidemiology Program Office. Her research interests include assessing attitudes toward uncertain longevity under expected utility and cumulative prospect theories and applying willingness-to-pay techniques to estimate societal values for preventing illness and death.

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Address for correspondence: Phaedra S. Corso, Division of Violence Prevention, National Center for Injury Prevention and Control, Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop K60, Atlanta, GA 30333, USA; fax: 770-488-1011; email: pcorso@cdc.gov

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# Antimicrobial Drug Prescriptions in Ambulatory Care Settings, United States, 1992–2000

Linda F. McCaig,* Richard E. Besser,* and James M. Hughes*

During the 1990s, as antimicrobial resistance increased among pneumococci, many organizations promoted appropriate antimicrobial use to combat resistance. We analyzed data from the National Ambulatory Medical Care Survey, an annual sample survey of visits to office-based physicians, and the National Hospital Ambulatory Medical Care Survey, an annual sample survey of visits to hospital emergency and outpatient departments, to describe trends in antimicrobial prescribing from 1992 to 2000 in the United States. Approximately 1,100–1,900 physicians reported data from 21,000–37,000 visits; 200–300 outpatient departments reported data for 28,000–35,000 visits; ~400 emergency departments reported data for 21,000–36,000 visits each year. In that period, the population- and visit-based antimicrobial prescribing rates in ambulatory care settings decreased by 23% and 25%, respectively, driven largely by a decrease in prescribing by office-based physicians. Antimicrobial prescribing rates changed as follows: amoxicillin and ampicillin, –43%; cephalosporins, –28%; erythromycin, –76%; azithromycin and clarithromycin, +388%; quinolones, +78%; and amoxicillin/clavulanate, +72%. This increasing use of azithromycin, clarithromycin, and quinolones warrants concern as macrolide- and fluoroquinolone-resistant pneumococci are increasing.

With the emergence of antimicrobial resistance (1–7), the use of antimicrobial drugs has increased in both inpatient (8) and outpatient settings (9,10). From 1995 through 1998, the overall proportion of isolates of *Streptococcus pneumoniae*, a community-acquired pathogen, that were resistant to three or more antimicrobial drug classes rose substantially (11), and high rates of antimicrobial use for upper respiratory tract infections are believed to be a major factor responsible for this increase. Although the overall antimicrobial prescribing rate by office-based physicians in the United States did not change from 1980 through 1992, the rate for children rose by 48% (12), and in 1992, antimicrobial agents were prescribed second in frequency behind cardiovascular-renal drugs in physicians' offices (13). Moreover, in the early 1990s, a sizable proportion of antibiotic prescriptions provided by office-based physicians to both children and adults were for colds, upper respiratory tract infections, and bronchitis, for which these drugs have little or no benefit (14,15).

During the 1990s, many organizations (e.g., the Centers for Disease Control and Prevention [CDC], American Academy of Pediatrics, American Academy of Family Practice, American Society of Microbiology, and Alliance for the Prudent Use of Antibiotics), conducted campaigns to promote appropriate antimicrobial use (16,17), defined by CDC as use that maximizes therapeutic impact while minimizing toxicity and the development of resistance. As a result of these and other efforts and increased media attention to the problem of antimicrobial resistance, antimicrobial prescribing for children seen in physician offices with respiratory infections decreased from 1989 through 2000 (18).

The objective of this study was to describe trends in antimicrobial prescribing at visits to office-based physicians, hospital outpatient departments, and hospital emergency departments in the United States. The results are based on a secondary data analysis using the 1992–2000 National Ambulatory Medical Care Survey (NAMCS) and National Hospital Ambulatory Medical Care Survey (NHAMCS).

## Methods

### Sample Design
NAMCS is a probability sample survey of office-based physicians in the United States conducted by CDC's National Center for Health Statistics. The U.S. Bureau of the Census has been responsible for field operations and data collection since NAMCS became an annual survey in 1989. A report describing sample design, sampling variance, and estimation procedures of the NAMCS has been published (19). NAMCS uses a three-stage probability sampling procedure. The first stage contains 112 geographic primary sampling units. The second stage consists of a probability sample of practicing nonfederally employed physicians (excluding those in the specialties of anesthesiology, radiology, and pathology) selected from the master files maintained by the American Medical Association and the American Osteopathic Association. Physicians selected to participate in NAMCS during a particular calendar year are not eligible to be selected again for at least another 3 years. The third stage involves selecting patient visits to the sample physicians during a randomly assigned 1-week reporting period in that year.

*Centers for Disease Control and Prevention, Hyattsville, Maryland, USA

NHAMCS is an annual probability sample survey of hospital outpatient departments and emergency departments in the United States, first conducted in 1992 by CDC's National Center for Health Statistics. The U.S. Census Bureau is responsible for field operations and data collection. A published report describes the plan and operation of NHAMCS (20). NHAMCS uses a four-stage probability sampling procedure. The first-stage sample contains the same 112 geographic primary sampling units as NAMCS. The second stage consists of a probability sample of nonfederal, short-stay or general hospitals with emergency departments, outpatient departments, or both, within the sampled primary sampling units. Hospitals are selected from a publicly available database of all hospitals in the United States. The third stage involves selecting emergency service areas within emergency departments and clinics within outpatient departments. Clinics are classified into six groups: general medicine, including internal medicine; surgery; pediatrics; obstetrics/gynecology; substance abuse; and other, which includes clinics such as psychiatry and neurology. Clinics where only ancillary services are provided, such as radiology, physical therapy, and nutrition, are excluded. The fourth stage consists of sampling patient visits within emergency departments or clinics during a randomly assigned 4-week reporting period in that year.

### Response Rates and Sample Size

From 1992 through 2000, the response rates ranged from 63% to 73% for NAMCS, 86% to 91% for NHAMCS outpatient departments, and 93% to 97% for NHAMCS emergency departments. The NAMCS response rate was defined as the number of eligible physicians who completed the survey plus the number of eligible physicians who saw no patients during the study period, divided by the sum of the numerator and the number of physicians who refused to participate. The NHAMCS response rate was defined as the number of completed cases divided by the sum of the numerator plus the number of case-patients who refused. For each year of the study, the number of participating NAMCS physicians ranged from 1,100 to 1,900, the number of NHAMCS outpatient departments, from 211 to 283, and NHAMCS emergency departments, from 375 to 425. The number of patient record forms completed each year for NAMCS ranged from 21,000 to 37,000, for outpatient departments, from 28,000–35,000, and for emergency departments, from 21,000–36,000. The number of antimicrobial patient record forms completed each year for NAMCS ranged from 2,000 to 4,200; for NHAMCS outpatient departments, 2,800–3,500; and for NHAMCS emergency departments, 3,700–6,600.

### Data Collection and Coding

The same patient record form is used for both the physician's office and outpatient department settings, whereas the emergency department form differs slightly to reflect the uniqueness of that setting. The form contains information about the visit, such as patient's date of birth and medications prescribed. Physician specialty was recorded for NAMCS during a personal interview with the physician. Physicians and hospital staff were instructed to record all new or continued medications ordered, supplied, or administered at the visit, including prescription and nonprescription preparations, immunizations, desensitizing agents, and anesthetics. From 1989 through 1994, up to five medications were recorded per visit, and from 1995 through 2000, up to six medications were listed per visit. Drugs were coded according to a classification system developed at the National Center for Health Statistics. A report describing the method and instruments used to collect and process drug information has been published (21). For this analysis, five drugs were assessed per visit. Since data on the route of administration were not collected, an attempt was made to delete topical preparations by reviewing trade names and excluding those intended for topical use (22–25). For this article, antimicrobial drugs were defined as drugs belonging to the following groups: quinolones (including nalidixic acid); azithromycin and clarithromycin; erythromycin; amoxicillin and ampicillin; amoxicillin/clavulanate; other penicillins; cephalosporins; trimethoprim-sulfamethoxazole; and tetracyclines.

### Rate Definitions

Two types of antimicrobial drug use rates were used in the analysis. The population-based rate was defined as the annual number of antimicrobial drugs recorded in the three ambulatory care settings divided by the civilian noninstitutional population of the United States. The population-based rate accounts for any changes that may have resulted in a patient being less likely to have visited an ambulatory care setting (e.g., an increase in telephone advice, education from a healthcare provider, or changes in insurance status). The visit-based rate was defined as the annual number of antimicrobial drugs recorded in the three ambulatory care settings divided by the annual number of ambulatory care visits in the United States. The visit-based rate reflects changes in prescribing behavior once a visit has occurred.

### Statistical Analysis

Data from NAMCS and NHAMCS samples were weighted to produce national estimates. From 1992 through 1994, NAMCS weight included three components: selection probability, nonresponse adjustment, and physician-population weighting ratio adjustment. In 1995, a fourth component, weight smoothing, was added. NHAMCS weight includes three components: selection probability, nonresponse adjustment, and ratio adjustment to fixed totals. SUDAAN statistical software was used for all statistical analyses (26). The standard errors used to calculate the 95% confidence intervals (CI) around the estimates took into account the complex sample designs of NAMCS and NHAMCS. All estimates in this analysis had <30% relative standard error (i.e., the standard error divided by the estimate expressed as a percentage of the estimate) and were based on 30 cases or more in the sample data. Significance of trends was based on a weighted least-

squares regression analysis at the 0.01 level of confidence (27).

## Results

From 1992 through 2000, the number of antimicrobial drug prescriptions in ambulatory care settings in the United States declined from 151 million (95% CI 132 to 169) to 126 million (95% CI 112 to 141), while the number of visits rose from 908 million (95% CI 842 to 975) to 1.0 billion (95% CI 0.9 to 1.1). The annual population-based rate of antimicrobial drug use decreased by 23% (from 599 [95% CI 524 to 673] antimicrobial drug prescriptions per 1,000 persons to 461 [95% CI 409 to 513]) (p<0.001), and the annual visit-based rate of antimicrobial drug use declined by 25% (from 166 [95% CI 152 to 179] antimicrobial drug prescriptions per 1,000 visits to 125 [95% CI 116 to 133]) (p<0.001) during the study period (Figure 1). All subsequent rates shown are visit-based rates. The antimicrobial prescribing rate at ambulatory care visits decreased in persons <15 years of age (–32%; p<0.001), 15–24 years (–9%; p=0.007), and 25–44 years of age (–17%; p<0.001). No trend was found among persons ≥45 years (p=0.03) (Figure 2). For children <15 years of age, antimicrobial prescribing rates decreased by 34% in physicians' offices (p<0.001) and by 13% in emergency departments (p<0.001), but no trend was observed in the prescribing rates in outpatient departments (p=0.17) (Figure 3). The physician's office was the only ambulatory care setting which experienced a decline in antimicrobial prescribing rates for persons ≥15 years (–24%; p<0.001), while an increasing trend was seen in outpatient departments (+35%; p=0.002), and no change was observed in emergency departments (Figure 4). For visits to physician offices, antimicrobial prescribing rates decreased for general and orthopedic surgeons (–45%; p<0.001), general and family practitioners (–34%; p<0.001), pediatricians (–33%; p<0.001), and dermatologists (–4%; p=0.006) (Table 1).

During the study period, the antimicrobial prescribing rate at all ambulatory care visits declined for amoxicillin and ampicillin (–43%;p<0.001), cephalosporins (–28%; p<0.001), and erythromycin (–76%; p<0.001) (Figure 5); the prescribing rate rose for azithromycin and clarithromycin (+388%; p<0.001), quinolones among persons ≥15 years (+78%; p<0.001), and amoxicillin/clavulanate among children <15 years (+69%; p<0.001) (Figure 6). Decreasing trends were also found for other penicillins (p<0.001), tetracyclines (p<0.001), and trimethoprim-sulfamethoxazole (p=0.009) (data not shown). Table 2 shows the rank order of the nine drug classes examined in 1992 compared with their order in 2000.

## Discussion

Our study found decreasing trends in both the population- and visit-based antimicrobial prescribing rates in ambulatory care settings from 1992 through 2000. The population-based prescribing rate provides the number of antimicrobial drugs used per person in the United States; we used this rate to assess changes over time that may be attributed to variations in visit-
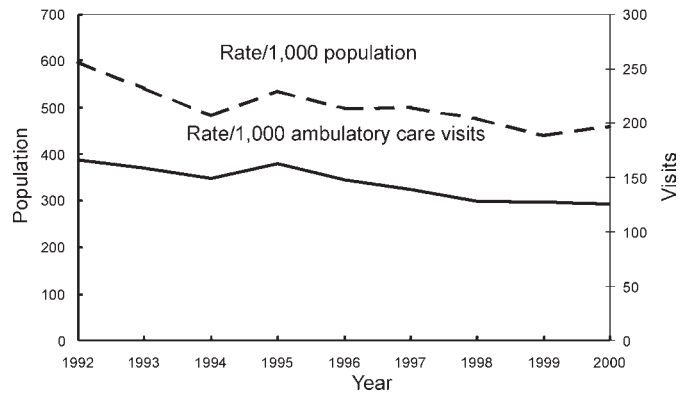


Figure 1. Trends in annual antimicrobial prescribing rates—United States, 1992–2000. Note: all trends shown are significant (p<0.001).

ing an ambulatory care setting. Declining population-based antimicrobial prescribing rates may be a result of several factors: a decrease in visits which, for example, may be due to a decrease in the incidence of a disease or changes in the patient's health insurance coverage; a decrease in prescribing, which may be the result of an increased understanding by the patient and/or healthcare provider of the impact of antimicrobial use, or both. Declining visit-based antimicrobial prescribing rates only reflect a change in prescribing behavior occurring at ambulatory care visits.

The decreasing trends in the antimicrobial prescribing rate found in this study for both children and adults seen in physicians' offices from 1992 through 2000 contrast with findings of a previous report that examined NAMCS data from 1980 through 1992. That report showed an increasing trend in antimicrobial prescribing for children and no trends for the older age groups (12). Although NAMCS data for children have been published previously in a slightly different format (18), showing the prescribing rates in all three settings is important to understanding practice patterns in ambulatory care. The findings suggest that efforts to promote appropriate antimicrobial use in physicians' offices may be effective.
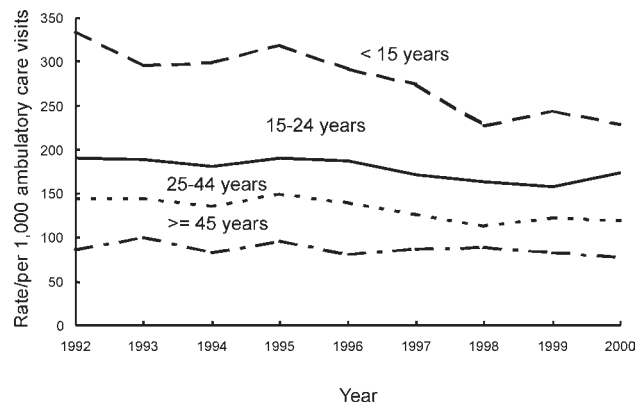


Figure 2. Trends in annual antimicrobial prescribing rates by age—United States, 1992–2000. Note: trend for visits by patients <15 years of age, p<0.001; for visits by patients 15–24 years, p=0.007; for visits by patients 25–44 years, p<0.001.
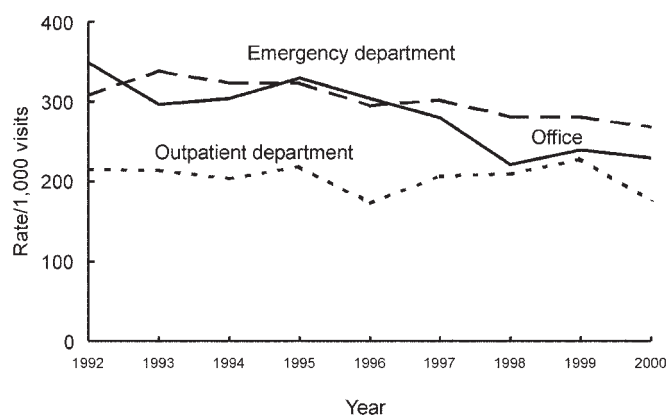
Figure 3. Trends in annual antimicrobial prescribing rates for persons <15 years of age by setting—United States, 1992–2000. Note: trend for office setting and emergency departments, p<0.001.

Increasing rates of use were observed for some of the new, more expensive, broad-spectrum antimicrobial agents, such as azithromycin and clarithromycin, quinolones, and amoxicillin/clavulanate. The large increase in the use of azithromycin and clarithromycin may be partially explained by the fact that clarithromycin was first mentioned in NAMCS and NHAMCS in 1992 and azithromycin in 1993. While these agents have been recommended for use in some patients with community-acquired pneumonia (28), cases of pneumonia are unlikely to account for this dramatic increase in their use. Fluoroquinolones and newer macrolides (azithromycin and clarithromycin) are rarely indicated as first-line therapy for other respiratory infections (29,30). The decrease in the use of amoxicillin and ampicillin could be a consequence of the 46% decrease in visits to physician offices for otitis media from 1989 through 2000 (18).

Antimicrobial use, whether appropriate or inappropriate, promotes antimicrobial resistance. The increasing use of azithromycin, clarithromycin, and fluoroquinolones warrants concern in light of the importance of these agents in the treat-ment of patients hospitalized with pneumonia, and the rise in macrolide- and fluoroquinolone-resistant pneumococci in many parts of the world (11,31–35). Making certain that the increasing use of these agents is clinically appropriate is important. While most efforts to date promoting appropriate antibiotic use have focused on reducing the use of antimicrobial agents for viral infections, future efforts should be directed towards ensuring that when antimicrobial agents are indicated, first-line or targeted therapy is employed.
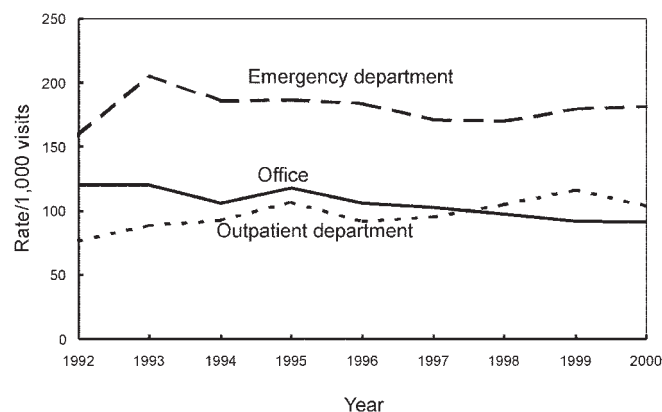


Figure 4. Trends in annual antimicrobial prescribing rates for persons ≥15 years of age by setting—United States, 1992–2000. Note: trend for office setting, p<0.001; trend for outpatient departments, p=0.002.

Decreasing trends in antimicrobial drug prescribing rates were found for office visits to pediatricians, general and family practitioners, dermatologists, and general and orthopedic surgeons. Interventions may need to be tailored differently to different settings (e.g., physician's office versus outpatient department versus emergency department) and physician specialty groups. In 2000, the American College of Physicians-American Society of Internal Medicine (ACP-ASIM) designated antimicrobial resistance as a focus for their continuing medical education conferences. The ACP-ASIM, together with

Table 1. Trends in annual antimicrobial drug prescribing rates at physicians' offices by specialty—United States, 1992–2000

| Physician specialty | No. of antimicrobial drug prescriptions/1,000 visits[a] | | | | | | | | | % change since 1992 |
| | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Pediatrics | 353 (310, 397) | 325 (276,374) | 302 (255,349) | 344 (304,384) | 340 (291,389) | 299 (262,336) | 218 (182,253) | 258 (202,314) | 235 (208,263) | −33[b] |
| General/family practice | 265 (232,298) | 226 (199,254) | 241 (216,267) | 231 (204,258) | 201 (178,225) | 207 (181,234) | 187 (164,209) | 188 (160,216) | 176 (148,204) | −34[b] |
| Otolaryngology | 182 (141,223) | 218 (177,259) | 181 (146,217) | 197 (153,241) | 179 (147,210) | 189 (122,256) | 169 (135,203) | 162 (97,227) | 166 (128,205) | −8 |
| Internal medicine | 139 (114,165) | 147 (117,178) | 143 (111,174) | 162 (137,187) | 147 (114,180) | 123 (97,149) | 142 (122,162) | 138 (104,173) | 116 (95,136) | −17 |
| Dermatology | 138 (110,167) | 149 (124,173) | 140 (114,166) | 134 (107,161) | 116 (97,136) | 106 (75,137) | 112 (83,141) | 92 (70,114) | 133 (110,157) | −4[b] |
| Urology | 118 (90, 145) | 129 (100,158) | 144 (117,172) | 158 (120,196) | 122 (85,159) | 153 (108,199) | 108 (84,133) | 131 (89,172) | 148 (123,172) | +26 |
| General/orthopedic surgery | 40 (26,54) | 39 (26,51) | 30 (18,42) | 30 (20,40) | 39 (24,54) | 44 (24,64) | 14 (7,21) | 28 (12,44) | 22 (14,30) | −45[b] |
| All others | 39 (28,49) | 51 (34,68) | 40 (31,49) | 50 (32,69) | 39 (26,51) | 37 (28,47) | 42 (30,53) | 30 (22,38) | 36 (27,45) | −6 |

[a]95% confidence interval.
[b]Trend is significant (p<.01).
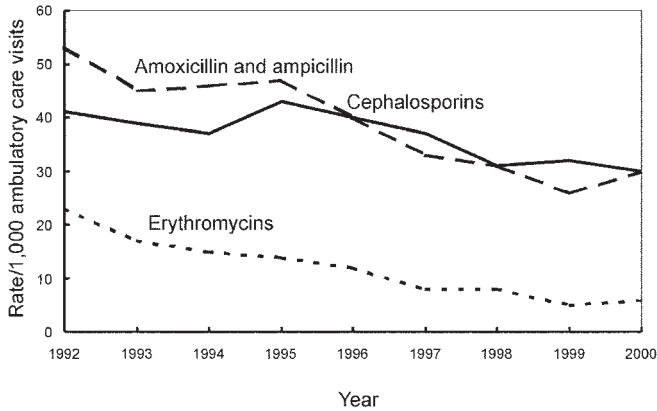
Figure 5. Trends in decreasing annual antimicrobial prescribing rates by drug class—United States, 1992–2000. Note: all trends shown are significant (p<0.001).

Table 2. Rank order of antimicrobial drug classes in ambulatory care settings, 1992 and 2000

| Antimicrobial drug class | 1992 | 2000 |
|---|---|---|
| Amoxicillin and ampicillin[a] | 1 | 1 |
| Cephalosporins | 2 | 2 |
| Erythromycin | 3 | 8 |
| Tetracyclines | 4 | 7 |
| Other penicillins | 5 | 9 |
| Trimethprim-sulfamethoxazole | 6 | 6 |
| Quniolones | 7 | 4 |
| Amoxicillin/clavulanate | 8 | 5 |
| Azithromycin and clarithromycin[b] | 9 | 3 |

[a]Excludes amoxicillin/clavulanate.

[b]Only clarithromycin was in the National Ambulatory Medical Care Survey and National Hospital Ambulatory Medical Care Survey drug databases in 1992.

CDC and the American Academy of Family Physicians and the Infectious Diseases Society of America, has published principles for appropriate prescribing for upper respiratory infections in adults (29). These principles will form the scientific basis for new campaigns to improve prescribing by clinicians who treat adults. Future analyses of NAMCS and NHAMCS data will show whether these activities result in changes in prescribing behavior similar to those seen for children.

The major limitation of our study is that the appropriateness of an antimicrobial prescription cannot be assessed in most instances because diagnosis is not linked to a particular drug. Patient visits in NAMCS or NHAMCS do not include telephone contacts; therefore, we could not determine whether a shift to telephone prescribing for antimicrobial agents occurred. However, we could assess whether prescribing had made a transition from physicians' offices to emergency departments or outpatient departments. A shift to other healthcare settings (at least for children <15 years of age) did not appear to occur because a decreasing trend was also found in emergency departments in addition to physicians' offices, and outpatient

departments did not show a trend. However, for adults, antimicrobial drug prescribing declined in physicians' offices, remained the same in emergency departments, and rose in outpatient departments, suggesting that a change in setting could have occurred.

The dynamics that influence antimicrobial prescribing are complex. In recent years, physicians have been receiving messages about the appropriate use of antimicrobial drugs from the medical literature, the media, health insurance companies, key opinion leaders, alternative medicine leaders, and patients (36). These messages appear to have been absorbed to some extent, as evidenced by the results shown in this article and the decline in antimicrobial prescribing in children seen in physicians' offices (18). However, the increasing use of azithromycin, clarithromycin, and quinolones evokes concern and requires additional study to determine their appropriateness. New efforts must be made to promote targeted agents as first-line therapy.

## Acknowledgments

Ms. McCaig is a health scientist who conducts national surveys on ambulatory healthcare utilization. Her research interests include antibiotic prescribing practices, emergency department use, and utilization of physician assistants and nurse practitioners in ambulatory–care settings.



Figure 6. Trends in increasing annual antimicrobial prescribing rates by drug class—United States, 1992–2000. Note: trend for amoxicillin/clavulanate prescribing among children<15 years of age, p=0.004; for quinolones among persons >15 years, p<0.001; for azithromycin and clarithromycin among all ages, p<0.001.

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Address for correspondence: Linda F. McCaig, Division of Health Care Statistics, National Center for Health Statistics, Centers for Disease Control and Prevention, 3311 Toldeo Road, Room 3409 (Mailstop P08), Hyattsville, MD 20782, USA; fax: 301-458-4302; email: lfm1@cdc.gov

# Dengue Fever in Travelers to the Tropics, 1998 and 1999

Heidi Lindbäck,*† Johan Lindbäck,† Anders Tegnell,† Ragnhild Janzon,† Sirkka Vene,† and Karl Ekdahl†

Dengue fever (DF) has become common in western travelers to the tropics. To improve the basis for travel advice, risk factors and dengue manifestations were assessed in 107 Swedish patients for whom DF was diagnosed after return from travel in 1998 and 1999. Patient data were compared with data on a sample of all Swedish travelers to dengue-endemic countries in the same years. Only three of the patients had received pretravel advice concerning DF from their physicians. Hemorrhagic manifestations were common (21 of 74 patients) but caused no deaths. Risk factors for a DF diagnosis were travel to the Malay Peninsula (odds ratio [OR] 4.95; confidence interval [CI] 2.92 to 8.46), age 15–29 years (OR 3.03; CI 1.87 to 4.92), and travel duration >25 days (OR 8.75; CI 4.79 to 16.06). Pretravel advice should be given to all travelers to DF-endemic areas, but young persons traveling to southern and Southeast Asia for >3 weeks (who constituted 31% of the patients in our study) may be more likely to benefit by adhering to it.

Dengue fever (DF) is an acute, self-limiting febrile disease of 2–7 days' duration, characterized by a sudden onset of fever and a variety of other symptoms such as severe headache, joint and muscular pain, retroorbital pain, and rash. Occasionally, hemorrhagic manifestations, such as skin hemorrhages, gum bleeding, epistaxis, menorrhagia, and gastrointestinal hemorrhage, occur. Dengue hemorrhagic fever (DHF) is a more severe disease with fever, hemorrhagic phenomena, thrombocytopenia, and plasma leakage caused by increased vascular permeability. In patients with DHF, a sometimes-fatal circulatory failure with hypovolemic shock, called dengue shock syndrome, can develop (1,2).

The dengue virus (formal name: *Dengue virus* [DENV]) belongs to the family Flaviviridae, which also includes yellow fever virus and Japanese encephalitis virus (2). Dengue virus has four serotypes, DENV-1 to DENV-4. Infection with one of these serotypes conveys life-long immunity but not cross-protective immunity to the other serotypes (2). Serologic analysis is difficult because of cross-reactivity between the viruses. However, together with clinical symptoms and travel history, dengue serology can yield a diagnosis (3). Humans are infected with dengue virus by the bite of infective *Aedes* mosquitoes. The most important vector is *Aedes aegypti* (1), which prefers to feed on humans during daylight hours. The incubation time is 3–14 days, most often 4–7 days (2).

DF is endemic in most countries in the tropical areas of southern and Southeast Asia, the Western Pacific regions, Central and South America, the Caribbean, and Africa (1,4). Transmission of DF increases during the rainy season (1,5).

With an increased travel to tropical countries (6), and an increased incidence of DF in these countries (1,7), DF has become the most common imported arbovirus disease in Sweden (8). In the absence of an effective vaccine, pretravel advice, mainly on protection against mosquito bites, is important to prevent the disease (2,7,9). Such advice should be focused on individual risk assessments, based on available epidemiologic data. The aim of this study was, in the light of changing travel patterns, to give an update on risk factors for DF in order to form the basis for pretravel advice.

## Patients and Methods

### Cases

The Department of Virology at the Swedish Institute for Infectious Diseases is the only laboratory in Sweden that performs dengue serology. Indirect immunofluorescence is used for the diagnosis. Antibody detection by indirect immunofluorescence has proved to be at least as reliable as hemagglutination inhibition for diagnosis of DF in Swedish patients (3,10). All cases with a positive dengue serologic test diagnosed at the Swedish Institute for Infectious Diseases in 1998 and 1999 were considered for inclusion in the study. Thus, 114 patients, 92 from 1998 and 22 from 1999, were identified. Seven patients were excluded from the study because of incorrect or missing data that prevented us from confirming age and sex. From the laboratory records, data for the remaining patients were obtained on age, sex, name of physician, country of infection, and the date when the patient became ill. A detailed questionnaire on the journey, symptoms, preventive measures, and pretravel advice was sent to the 107 case-patients through their physicians.

### Control Group

A control population consisting of travelers to dengue-endemic countries was used (Åre Marknadsfakta AB, Åre, Sweden). The Åre dataset is a commercial database, based on a randomized selection of 2,000 members of the Swedish population every month. These persons are interviewed by telephone with questions on recent overnight travel outside

*Uppsala University, Uppsala, Sweden; and †Swedish Institute for Infectious Disease Control, Solna, Sweden

Sweden. Data are then extrapolated to estimate the total number of Swedish travelers abroad. No data on any illness are available from this dataset. The data are presented as journeys per principal country or geographic area, categorized by age, sex, length of journey, and purpose for travel (business or leisure). A total of 4,217 persons with overnight travel abroad were recorded in the Åre database for 1998 and 1999; 292 had traveled to dengue-endemic countries or regions, defined as those reporting one or more dengue cases to the World Health Organization (WHO) in 1998 (11). These 292 persons were included as controls.

### Statistics

Odds ratios (ORs) with the corresponding 95% confidence interval (CI) were calculated as relative risk measures to assess the risk factors for being diagnosed with DF. These ORs should be interpreted as the odds of being exposed to a risk factor among the cases divided by the odds of being exposed to that risk factor among the controls, given that only traveling to areas with risk of DF is under consideration. To study whether risk factors were confounded, stratified analyses were performed and Mantel-Haenszel ORs calculated. We performed all analyses with Epi Info 6.04 software (Centers for Disease Control and Prevention, Atlanta, GA).

### Results

#### Cases

Most cases (95 of 107) were investigated at departments of infectious diseases throughout Sweden. Of the original 107 patients who received a questionnaire, 74 (69%) responded. DF was diagnosed in 59 responders and 29 nonresponders in 1998 and in 15 responders and 4 nonresponders in 1999. Of the 74 responders, 40 (54%) were women and 34 men, compared with 13 (39%) women and 20 men among the nonresponders (p=0.16). The responders were of similar age (median 28 years; range 19–60 years), as the nonresponders (median age 28 years; range 8–52 years). Age distribution did not differ between the different countries of infection.

#### Country of Infection

The country where the person was staying 3–14 days before becoming ill with DF was considered the country of infection. Data on country of infection were available for 105 of the 107 patients. Most patients (75 of 105; 71%) were infected in Thailand. The remaining patients were infected in 15 different countries (Table 1). The DF risk per 1,000 travelers is shown in Table 2, in comparison with the number of cases reported to WHO in 1998 (11).

In the Figure, month of disease onset (combined for 1998 and 1999) is shown for the 75 travelers to Thailand. For comparison, the mean seasonal variation for indigenous cases in Thailand in the years 1987–1991 (5) is also shown.

Table 1. Country of infection for dengue fever cases

| Country of infection | All cases | Responders | Nonresponders |
|---|---|---|---|
| Thailand | 75 | 50 | 25 |
| Philippines | 5 | 5 | 0 |
| India | 4 | 3 | 1 |
| Caribbean Islands (unspecified) | 4 | 3 | 1 |
| Malaysia | 3 | 3 | 0 |
| Sri Lanka | 2 | 2 | 0 |
| Central America (unspecified) | 2 | 2 | 0 |
| Indonesia | 2 | 0 | 2 |
| Nepal | 1 | 1 | 0 |
| Singapore | 1 | 1 | 0 |
| Cambodia | 1 | 0 | 1 |
| Laos | 1 | 1 | 0 |
| Vietnam | 1 | 0 | 1 |
| Nicaragua | 1 | 1 | 0 |
| Colombia | 1 | 1 | 0 |
| Tanzania | 1 | 1 | 0 |
| Unknown | 2 | 0 | 2 |
| Total | 107 | 74 | 33 |

[a]Divided by responders and nonresponders to travel questionnaires.

#### Length of Stay

The median length of time spent abroad for the 74 case-patients was 30 days (range 11–496 days). The length of stay in the country of infection was less in some cases, since one person could have traveled to more than one country. The mean length of stay did not differ between the different countries of infection.

#### Risk Factors for DF

With the Åre sample used as controls, ORs for a diagnosis of DF were calculated for the risk factors of age, country of infection, and length of stay (Table 3). Risk factors for DF were travel to the Malay Peninsula (OR 4.95; 95% CI 2.92 to 8.46), age 15–29 years (OR 3.03; CI 1.87 to 4.92), and travel duration >25 days (OR 8.75; CI 4.79 to 16.06). OR for travel to the Malay Peninsula was basically unchanged after stratification by age group (OR 4.48) or length of stay (OR 4.41). When stratified for travel versus no travel to the Malay Peninsula, the results for length of stay remained unchanged, as did the results for age group. The Åre database did not provide us with cross-classified information about age group and length of stay so those factors could not be studied together. Unadjusted ORs are shown in Table 3.

#### Prophylaxis against Mosquito Bites

Bed nets as prophylaxis against mosquito bites were used regularly by 17 of the DF patients, irregularly by 14 patients, and never by 42 patients. Mosquito repellents were used regularly by 13 patients, irregularly by 37 patients, and never by 23 patients. One person did not answer the questions on prophylaxis.

#### Information on DF

Fourteen of 74 patients stated that they had been informed about DF before the journey, 5 could not remember, and the

Table 2. Geographic area of DF infection, Swedish travelers to these countries, DF risk, and number of cases and incidence of DF/DHF per 100,000 inhabitants[a,b]

| Geographic area of infection[c] | Dengue patients | Estimate of all Swedish travelers | DF risk/100,000 travelers | DF/DHF cases reported to WHO in 1998 | DF/DHF incidence rate/100,000/y |
|---|---|---|---|---|---|
| India with its neighboring countries[d] | 7 | 12,000 | 58 | 707 | <1 |
| Malay Peninsula[e] | 79 | 264,000 | 30 | 158,901 | 180 |
| China | 0 | 32,000 | 0 | 15 | <1 |
| The rest of Asia, excluding Japan[f] | 3 | 33,000 | 9 | 181,847 | 130 |
| Australia and Pacific Islands[g] | 7 | 103,000 | 7 | 132,126 | 40 |
| Caribbean Islands | 4 | 165,000 | 2 | 24,545 | 121 |
| Central America | 3 | 55,000 | 5 | 67,403 | 49 |
| South America | 1 | 54,000 | 2 | 641,299 | 217 |
| West Africa[h] | 0 | 16,000 | 0 | No data | No data |
| East Africa[i] | 1 | 17,000 | 6 | No data | No data |
| South Africa[j] | 0 | 38,000 | 0 | No data | No data |
| Total | 105 | 789,000 | 13 | 1,203,831 | 37 |

[a]DF, dengue fever; DHF, dengue hemorrhagic fever; WHO, World Health Organization.
[b]As reported to WHO. Data are combined figures for 1998 and 1999, except for information from the WHO report, which covers 1998 (11). The DF/DHF incidence calculations are based only on those countries in the regions reporting DF/DHF cases to WHO.
[c]Categorized according to the Åre database.
[d]India, Afghanistan, Bangladesh, Bhutan, Nepal, Pakistan, Sri Lanka.
[e]Malaysia, Singapore, Thailand.
[f]Brunei, Cambodia, Lao People's Democratic Republic, Maldives, Myanmar (Burma), North Korea, South Korea, Taiwan, Viet Nam.
[g]Australia, Indonesia, New Zealand, Papua New Guinea, Philippines, and other Pacific islands.
[h]Benin, Burkina Faso, Ivory Coast, The Gambias, Ghana, Guinea, Guinea-Bissau, Cape Verde, Liberia, Mali, Mauretania.
[i]Ethiopia, Kenya, Somalia, Tanzania.
[j]Angola, Lesotho, Madagascar, Malawi, Mocambique, Namibia, South Africa, Zambia, Zimbabwe.

remaining 55 patients stated they had not received any information. Three of the 14 informed patients had received the information from their doctor; the rest had received it from friends, the Internet, or travel literature. Of the 74 patients, 23 were aware that DF occurred at the country of infection.

## Clinical Data

Thirty patients visited a physician in the country where they became ill, as well as after returning to Sweden. All patients had fever, and 58 patients (78%) had headache. The other non-hemorrhagic symptoms noted were retroorbital pain (n=23; 31%), musculoskeletal and joint pain (n=54; 73%), conjunctivitis (n=17; 23%), rash (n=46; 62%), gastrointestinal complaints (n=12; 16%), neurologic complaints (n=8; 11%), psychologic complaints (n=3; 4%), alopecia (n=2; 3%), and respiratory complaints (n=1; 1%). One or more hemorrhagic manifestations consistent with DHF (12) were observed in 21 patients, including petechiae (n=12; 16%), epistaxis (n=9; 12%), hematuria (n=2; 3%), hematemesis (n=2; 3%), melena (n=2; 3%), menorrhagia (n=2; 3%), gum bleeding (n=2; 3%), and internal bleeding (n=1; 1%). The one patient with internal bleeding stated that he was treated for DHF at a hospital in Bangkok. He had lived in Laos earlier in his life but had no knowledge of previous DF.

## Discussion

The number of Swedes and other westerners traveling to DF-endemic areas has steadily increased. The patients in our study only included those Swedish patients with DF who, after returning from their travels, visited a physician in Sweden who made a diagnosis on the basis of a positive dengue serologic test. Questionnaires were sent out with the help of the patients'

physicians. Whether the questionnaires ever reached the nonresponders is not known. Responders were mostly women, and nonresponders were mostly men. Age and country of infection were similar between the groups, except for in travelers to India and its neighboring countries (Afghanistan, Bangladesh, Bhutan, Nepal, Pakistan, and Sri Lanka) and to Central America, where fewer cases occurred in nonresponders than in responders. We do not think that these differences significantly affect the results and conclusions from this study. Since not all persons with symptoms visited a physician, or only did so abroad, our case-patients probably represent a small fraction of the Swedish travelers ill with DF in the 2 study years. In 1998, DF was diagnosed in considerably more patients in Sweden (92 patients) than during the previous years, 1991–1997 (median 24; range 0–45) (8). Similarly, in 1998 more than twice as many cases were reported to WHO than the average for the 3 preceding years.
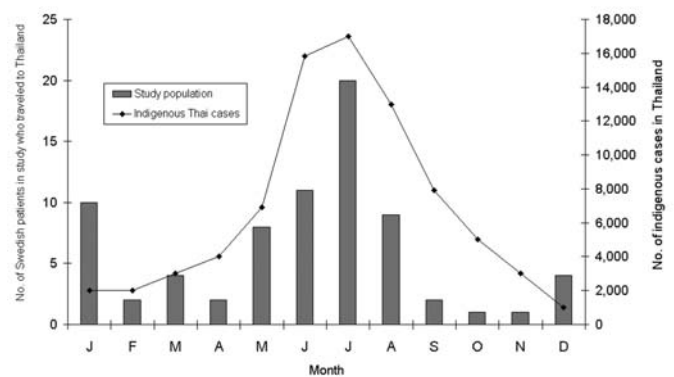


Figure. Month of disease onset in 75 Swedish patients with dengue fever or dengue hemorrhagic fever infected in Thailand, compared with the mean number of indigenous cases in Thailand per year, 1987–1991 (11).

Table 3. Odds ratios for diagnosis of dengue fever, calculated for possible risk factors

| Risk factor | Ratio in cases | Ratio in controls | Odds ratio | 95% CIª |
|---|---|---|---|---|
| Geographic area | | | | |
|   India with neighbors | 7/105 | 7/292 | 2.91 | 0.89 to 9.50 |
|   Malay Peninsula | 79/105 | 111/292 | 4.95 | 2.92 to 8.46 |
|   China | 0/105 | 17/292 | 0.00 | 0.00 to 0.65 |
|   The rest of Asia | 3/105 | 15/292 | 0.54 | 0.10 to 1.98 |
|   Carribean | 4/105 | 40/292 | 0.25 | 0.06 to 0.72 |
|   Central America | 3/105 | 17/292 | 0.48 | 0.11 to 1.77 |
|   South America | 1/105 | 21/292 | 0.12 | 0.00 to 0.79 |
|   West Africa | 0/105 | 6/292 | 0.00 | 0.00 to 2.60 |
|   East Africa | 1/105 | 4/292 | 0.69 | 0.01 to 7.11 |
|   South Africa | 0/105 | 11/292 | 0.00 | 0.00 to 1.09 |
|   Australia and Pacific Islands | 7/105 | 43/292 | 0.41 | 0.15 to 0.97 |
| Age groups (y) | | | | |
|   0–14 | 2/107 | 14/292 | 0.38 | 0.04 to 1.69 |
|   15–29 | 58/107 | 82/292 | 3.03 | 1.87 to 4.92 |
|   30–44 | 33/107 | 79/292 | 1.20 | 0.72 to 2.01 |
|   ≥45 | 14/107 | 117/292 | 0.23 | 0.12 to 0.43 |
| Sex | | | | |
|   Male | 54/107 | 154/292 | 0.91 | 0.57 to 1.46 |
| Length of travel (nights) | | | | |
|   0–10 | 0/74 | 68/292 | 0.00 | 0.00 to 0.17 |
|   11–17 | 16/74 | 149/292 | 0.26 | 0.14 to 0.50 |
|   18–24 | 13/74 | 31/292 | 1.79 | 0.83 to 3.82 |
|   ≥25 | 45/74 | 44/292 | 8.75 | 4.79 to 16.06 |

ªCI, confidence interval.

For the diagnosis, we used antibody detection by indirect immunofluorescence. As with other antibody assays, such as the hemagglutination inhibition method, serologic cross-reactions with other flaviviruses cannot be ruled out. However, in addition to their positive serologic results, all patients had a history of signs and symptoms compatible with DF.

The data presented in this study only represent Swedish travelers. The travel patterns of persons from other western countries may differ. However, since we have adjusted for age, sex, and length of travel, and the risk estimates are related to the number of Swedes traveling to different countries, the main conclusions should also be valid for travelers from other countries to those areas for which we have enough power to detect elevated risks.

No data on travel-related illnesses in the controls were available from the Åre database. Therefore we cannot exclude the possibility that the control group could have included persons who actually had DF during their travel. However, all calculations are based on the odds of DF's being diagnosed in Sweden after the traveler returned home, not on the odds of becoming ill with DF (for which we do not have any data). If we consider the small number of diagnosed cases and controls in relation to the estimated total number of travelers (circa 1:2,000), any single person being included both as a patient and as a control would be unlikely.

Large discrepancies exist between the calculated risks for the Swedish travelers to become ill with DF in different regions and the incidence rates of cases reported to WHO. Several factors might explain these variations, including the small number of Swedish DF patients, different sensitivity of the surveillance and reporting systems in different countries, and the classification of regions in the Åre database, which sometimes includes both dengue-endemic and nonendemic countries in a region. Some of the non–DH-endemic countries thus included do not have many tourists (e.g., Afghanistan, North Korea, Liberia), but the inclusion of non–DH-endemic countries such as South Korea, New Zealand, Chile, Argentina, and Uruguay may have diluted the denominator sufficiently to give falsely low ORs for the rest of Asia, South America, and Australia and Pacific Islands. Furthermore, DF is endemic in only limited areas of China and Australia. The risk of contracting DF in the disease-endemic parts of these countries is therefore substantially higher than reflected by the low ORs in this study.

The main risk factors for DF were travel to the Malay Peninsula (mainly Thailand), age 15–29 years, and travel duration >25 days. The Åre database is based on a relatively small sample of travelers from each country; the results for other single-risk countries is therefore more uncertain.

In 1998, many countries in Asia had unusually high levels of DF and DHF (13). That year, Thailand reported >126,000 cases of DF and DHF to WHO, compared with 99,000 cases in 1997, and 38,000 cases in 1996 (11). The DF epidemic in Asia in 1998 has probably also affected the odds for our case-patients' being infected in these countries. The results might have been different if the study had been performed in a nonepidemic year.

Most Swedish travelers to Thailand were infected during May to August, with a peak in July, a pattern that agrees with that of indigenous cases in Thailand in 1987 to 1991. The Swedish cases also peaked in December and January, a finding

that does not agree with the indigenous Thai cases. Most likely, this second peak among the Swedes reflects the travel pattern of many tourists who traveled to Thailand during the Christmas and New Year's holiday. A similar two-peak seasonal pattern was also observed among Israeli DF patients returning from Thailand in 1998 (14).

The general symptoms in the travelers with DF agreed with symptoms previously described (1,2). Many patients had clinical hemorrhagic manifestations, consistent with DHF (21/74). WHO's case definition for DHF includes thrombocytopenia as well as plasma leakage. Since we did not have access to laboratory test results, we cannot say how many of the patients fulfilled WHO's DHF case criteria. Patients with hemorrhagic manifestations may be more inclined to seek medical care than other DF patients, which would explain our findings.

In conclusion, DF is an important infection threatening travelers to disease-endemic areas. In the absence of available vaccines, pretravel advice on mosquito protection is important when attempting to reduce the number of DF cases in travelers. Only 3 of 74 case-patients in our study had received pretravel advice on DF from their doctor. Such advice should be given to all travelers to DF-endemic areas, but young person traveleing to southern and Southeast Asia for >3 weeks (31% of the patients in our study) may be more likely to benefit by adhering to it. This advice should also be given to travelers going to areas where DF is endemic, even when the disease is not in season.

Dr. Lindbäck is a physician with a keen interest in epidemiology and travel medicine. This analysis was conducted during her graduate studies.

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Address for correspondence: Karl Ekdahl, Department of Epidemiology, Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden; fax: +46-8 300 626; email: karl.ekdahl@smi.ki.se

## Instructions for Infectious Disease Authors

### Research

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. Report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").



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# Risk for Severe Group A Streptococcal Disease among Patients' Household Contacts

Katherine A. Robinson,* Gretchen Rothrock,†‡ Quyen Phan,§ Brenda Sayler,¶ Karen Stefonek,#
Chris Van Beneden,* and Orin S. Levine* for the Active Bacterial Core
Surveillance/Emerging Infections Program Network

From January 1997 to April 1999, we determined attack rates for cases of invasive group A streptococcal (GAS) disease in household contacts of index patients using data from Active Bacterial Core Surveillance sites. Of 680 eligible index-patient households, 525 (77.2%) were enrolled in surveillance. Of 1,514 household contacts surveyed, 127 (8.4%) sought medical care, 24 (1.6%) required hospital care, and none died during the 30-day reference period. One confirmed GAS case in a household contact was reported (attack rate, 66.1/100,000 household contacts). One household contact had severe GAS-compatible illness without confirmed etiology. Our study suggests that subsequent cases of invasive GAS disease can occur, albeit rarely. The risk estimate from this study is important for developing recommendations on the use of chemoprophylaxis for household contacts of persons with invasive GAS disease.

Group A streptococcus (GAS) causes a wide range of illnesses from noninvasive disease such as pharyngitis and pyoderma (1,2) to more severe invasive infections (e.g., bacteremia, pneumonia, and puerperal sepsis) (3,4). In the 1980s, invasive GAS infections received increasing attention from the medical community and the public because of necrotizing fasciitis (NF) (5,6) and the emergence of streptococcal toxic shock syndrome (STSS) (7–10). Based on results of the Active Bacterial Core Surveillance (ABCs)/Emerging Infections Program network, a population-based surveillance system, the Centers for Disease Control and Prevention (CDC) estimates that, in 1999, the annual invasive GAS incidence was 3.5 cases per 100,000 population, yielding approximately 9,400 cases and 1,200 deaths in the United States that year (11).

The severity of GAS disease, coupled with a number of case clusters reported in communities and families (12–14) and several anecdotal reports of subsequent cases of invasive GAS infection in close contacts, causes concerns about the spread of disease among close contacts and questions about whether chemoprophylaxis to prevent illness in close contacts is warranted. Using data from active surveillance in Ontario, Canada,

where the baseline rate of sporadic invasive GAS disease was 2.4 per 100,000 population (pers. comm.), investigators estimated that the attack rate of disease among household contacts of patients with invasive GAS disease was higher than the rate of invasive disease among the general population (294.1/100,000 population) (3).

In October 1995, the Working Group on Prevention of Invasive GAS Infections, composed of streptococcal experts from a variety of clinical and public health organizations, CDC, and various academic institutions, held a meeting to examine existing data and to determine if these data were sufficient to recommend widespread use of chemoprophylaxis to prevent subsequent invasive GAS disease among close contacts of index patients. Four specific criteria were used (15): severity of disease (16–19), virulence of the strain (18,20–23), increased risk for subsequent disease, and availability of an effective chemoprophylaxis regimen. Both the severity of invasive GAS disease and the virulence of GAS strains had been well documented. However, at that time, limited data existed regarding the risk for subsequent GAS disease among household contacts and an optimal regimen for chemoprophylaxis.

The working group concluded that a single study with four case-pairs was inadequate for establishing national recommendations for chemoprophylaxis for subsequent invasive GAS illness and emphasized the need for additional data on the risk of subsequent GAS disease among household contacts (15). We conducted surveillance to quantify the subsequent attack rates for both confirmed invasive GAS disease and severe GAS-compatible disease with no known etiology among household contacts in four geographic areas in the United States.

## Methods

### Identification of Index Patients

Cases of invasive disease attributed to GAS were identified through ABCs from January 1, 1997, to April 30, 1999. Active, population-based surveillance for laboratory-confirmed GAS infections occurred in four areas: the states of Connecticut and Minnesota; the San Francisco Bay area, California (three counties); and Portland, Oregon, (three counties). The aggregate population in 1998 was 12.1 million, or 4.5% of the U.S. population.

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †California Department of Health Services, Oakland, California, USA; ‡University of California, Berkeley, California, USA; §Connecticut Department of Public Health, Hartford, Connecticut, USA; ¶Minnesota Department of Health, Minneapolis, Minnesota, USA; and #Oregon Department of Human Services, Portland, Oregon, USA

Invasive GAS disease was defined as the isolation of *Streptococcus pyogenes* in a surveillance area resident from a normally sterile site (e.g., blood or cerebrospinal fluid) or from a wound (when accompanied by STSS or NF). Surveillance personnel reviewed records of all 208 clinical laboratories in the participating ABCs areas every 6 months to verify completion of case ascertainment. All available sterile site isolates were sent to CDC for confirmation and further microbiologic testing (e.g., *emm*-typing) (24).

A GAS index patient was defined as the person with the first invasive GAS infection in a household. A nosocomial GAS case was defined as a case-patient with a date of first positive culture obtained ≥2 days after admission to hospital. An institutional GAS case was defined as a case-patient who resided in a nursing home, jail, long-term skilled-care facility, or other long-term care institution.

### Identification of Eligible Households of Index Patients

Surveillance personnel contacted the households of all index patients to determine study eligibility. We restricted eligibility to households of index patients with community-acquired GAS infections. We excluded households of nosocomial, institutionalized, and homeless GAS index patients in addition to households of index patients who lived alone or were without phones. To reduce the effect of recall bias, we excluded households from which the case was not identified within 120 days of the culture date.

### Collection of Information on Household Contacts

We defined a household contact as a person who regularly spent 50% of nights or ≥24 h in a household with the index patient during the week before the index patient's date of culture. The index patients (or appropriate adult surrogates) of eligible households were interviewed by telephone within 31 to 120 days after the index patient's date of culture. Information collected on all household contacts included age, gender, underlying conditions, and relationship to the index patient. Study personnel also identified all household contacts who had sought medical care for any reason, been hospitalized, or died during the reference period.

Surveillance personnel abstracted the medical charts of all household contacts who had sought medical care, using a standardized data collection form to determine the types of visits, chief complaints, diagnostic tests results, type and duration of antibiotic use, and discharge diagnoses. All available sterile site GAS isolates from household contacts were collected and sent to CDC for confirmation and molecular testing.

We defined the study reference period as the 30 days after the index patient's date of GAS culture. A confirmed case of subsequent invasive GAS disease was defined as isolation of GAS from a household contact collected from a normally sterile site (or from a wound when accompanied by NF or STSS) within the study reference period. A probable case of subsequent severe disease was defined as a GAS-compatible illness resulting in hypotension, hospitalization, or death within the study reference period in a person from whom GAS was not isolated and for whom other infectious causes of disease were ruled out.

### Analysis

Analysis was performed by using SAS software, version 6.12 (SAS Institute, Inc., Cary, NC) and Epi Info, version 6.04c (CDC, Atlanta, GA). Attack rates (number of subsequent cases of invasive or severe GAS disease divided by number of household contacts, expressed as subsequent cases per 100,000 household contacts) were calculated for subsequent GAS disease among household contacts. We then compared the attack rate using only confirmed subsequent cases of invasive GAS disease to the sporadic incidence rate for invasive GAS disease among the general population to determine the increase in risk for subsequent GAS disease among household contacts. Exact 95% confidence intervals for the risk for subsequent GAS disease among household contacts were determined by using binomial distribution.

### Results

During the study period, 1,063 index patients with invasive GAS disease were identified, ranging in age from <1 year to 99 years (median, 48 years of age). The elderly (age ≥65 years of age) accounted for nearly one third (31.4%) of the invasive GAS cases. Most index patients had cellulitis with bacteremia (36.8%) or bacteremia with no focal point of infection (25.9%). Thirteen percent of the index patients had NF (6.5%), STSS (4.6%), or both (2.0%). Diabetes mellitus and alcohol abuse were the two most frequent medical conditions among patients with invasive GAS disease. Less than 5% of the index patients were infected with HIV.

Of the 1,063 households with index patients, 680 (64.0%) were eligible for the study. Ineligible households included those with index patients who had institutional infections (n=106, 10.0%), lived alone (n=106, 10%), had no telephone (n=42, 4.0%), or had nosocomial infections (n=37, 3.5%). Fifty-two (4.9%) of the index patients were homeless. Some households (n=36, 3.4%) were not eligible because the case was identified >120 days after the culture date. Of the 680 eligible index-patient households, 525 (77.2%) were enrolled. Eligible households not enrolled included those that could not be contacted (n=120, 17.6%) and those that refused to participate (n=24, 3.5%). Eleven households (1.6%) were not enrolled because of other reasons, primarily language barriers.

From the 525 enrolled households, 1,514 household contacts were identified and investigated (Table 1). Over half of the contacts were female (54%). The age distribution among the contacts was ≤93 years of age (median age, 29 years); 38.7% of contacts were children <18 years of age. Twelve percent of the household contacts (n=181) reported antibiotic use during the reference period. Approximately 9% (n=130) of the household contacts reported at least one underlying medical condition; the most common were chronic lung disease (3.0%) and congestive heart failure (2.6%).

Table 1. Demographic and clinical features of household contacts of invasive group A streptococcus index patients[a]

| Demographic or clinical feature | No. of household contacts[b] | Proportion of all household contacts (%) |
|---|---|---|
| Age (in y)[c] | | |
| 0–4 | 177 | 11.9 |
| 5–17 | 398 | 26.7 |
| 18–34 | 324 | 21.7 |
| 35–49 | 290 | 19.5 |
| 50–64 | 157 | 10.5 |
| ≥65 | 145 | 9.7 |
| Sex[d] | | |
| Male | 697 | 46 |
| Female | 810 | 54 |
| Underlying medical condition | | |
| Chronic lung disease | 46 | 3.0 |
| Congestive heart failure | 39 | 2.6 |
| Insulin-dependent diabetes | 32 | 2.1 |
| Cancer (except skin) | 23 | 1.5 |
| Other immunocompromising conditions[e] | 18 | 1.2 |
| Liver disease | 11 | 0.7 |
| Chronic kidney disease | 6 | 0.4 |

[a]N=1,514.
[b]Household contacts are counted more than once if multiple conditions exist.
[c]Age was missing for 23 household contacts.
[d]Sex was unknown for seven household contacts.
[e]Includes HIV infection, AIDS, intravenous drug use, chemotherapy for cancer, steroid use for other conditions such as recent organ transplant, or any illness from excessive use of alcohol.

Of the 1,514 household contacts, 127 (8.3%) sought medical care or were hospitalized during the reference period. No household contacts died during the reference period. Of the 127 household contacts who visited a physician, 104 (81.9%) reported having symptoms; however, 23 (18.1%) were asymptomatic at the time of their visit. Twenty of the asymptomatic household contacts reported visiting the physician because a family member had been ill with invasive GAS infection. Of the 104 symptomatic household contacts, infectious illness was diagnosed in 62 (59.6%). The diagnosis for most of these contacts was based on clinical evidence of streptococcal pharyngitis (n=10), obtained with a positive rapid strep test (n=36) or a positive throat culture (n=5). Eight cutaneous infections, one case of pneumonia documented by x-ray with no positive culture, and two clinically diagnosed cases of pneumonia were diagnosed in contacts. Of the 23 asymptomatic household contacts, 15 (65.2%) had evidence of GAS in the throat from a rapid strep test (n=13) or positive throat culture (n=2). Twenty-four household contacts required hospital care for various reasons during the reference period (13 hospital admissions and 11 emergency room visits).

During the study period, we identified one confirmed subsequent case of invasive GAS disease and one probable subsequent severe GAS disease in household contacts (Table 2). Both cases were diagnosed in immediate family members and resulted in hospitalization. The index patient in the one confirmed case-pair was a 76-year-old woman who was hospitalized with cellulitis and had a positive blood culture for GAS. The contact was her 69-year-old husband, who was hospital-

ized with cellulitis that progressed to NF 15 days after the index patient's culture date. A surgical specimen grew GAS, but the isolate was not available for confirmation or further testing by CDC. Both patients had underlying medical conditions.

The probable case-pair included an infant daughter and her father. The index patient was a 2-month-old girl hospitalized with GAS bacteremia with no focal point of infection. Her 39-year-old father was hospitalized 19 days after his daughter's date of culture; he had erysipelas accompanied by fever and hypotension (systolic blood pressure 86 mM Hg); a single blood culture was negative for GAS. He was hospitalized for 2 days and given intravenous antibiotics at home for 14 days. Neither the infant nor her father had underlying medical conditions.

We compared the attack rates of subsequent GAS disease in household contacts for this study to the Ontario, Canada, study (3). The attack rate of our study, using only confirmed cases of subsequent disease from ABCs, was 66.1 per 100,000 household contacts (95% confidence intervals [CI] 2 to 367). When both confirmed and probable cases of subsequent disease were used, the attack rate was 132.1 per 100,000 household contacts (95% CI 16 to 476); an estimate that remains lower than that measured among the Canadian study population.

## Discussion

During the 2-year study period in a population of 12.1 million, we identified one confirmed subsequent case of invasive GAS disease, resulting in an estimated risk of 66.1 per 100,000 household contacts. This attack rate represents an increased risk for disease among household contacts of index patients when compared to the annual incidence rate of sporadic invasive GAS disease in the United States (average rate 3.5/100,000 population, 1995–1999) (16). Although the risk estimate from this study is lower than the risk previously reported from surveillance in Canada, both risk estimates have extremely wide confidence intervals.

Our study has several strengths, including the large defined population base in four geographically diverse regions in the United States that participated in laboratory-based surveillance. The methods and completeness of case ascertainment of invasive infections for the ABCs system are well established. Also, the charts of all household contacts who reported seeking medical care during the 30-day reference period were reviewed for invasive or severe GAS infections.

The baseline rate of sporadic invasive GAS disease in this U.S. study was higher than that observed in the Canadian population, while the risk for subsequent GAS disease was lower than found in the Toronto study. Given the wide confidence intervals, a comparison of the risk estimate of subsequent infections between the two studies is not warranted. Further complicating a comparison of the studies are differences in physician management and frequency of blood culturing, factors that may affect the reported rate of sporadic invasive GAS disease.

Our study was limited in the lack of information on the use of chemoprophylaxis. We did not directly ask the household

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Table 2. Confirmed and probable subsequent invasive group A streptococcus disease case-pairs, Active Bacterial Core Surveillance (ABCs)

| Case-pair status | Case status | ABCs area | Sex | Age (in y) | Interval (d) | Diagnosis | GAS culture results | Underlying condition | Hospitalized? |
|---|---|---|---|---|---|---|---|---|---|
| Confirmed | Index | CA | Female | 76 | — | Cellulitis | Blood + | COPD, CHF | Yes |
| | Household Contact | | Male | 69 | 15 | Necrotizing fasciitis | Tissue + Blood – | Venous insufficiency | Yes |
| Probable | Index | CT | Female | 0 | — | Bacteremia | Blood + | None | Yes |
| | Household contact | | Male | 39 | 19 | Erysipelas | Blood – | None | Yes |

[a]GAS, group A streptococcus; Active Bacterial Core Surveillance (ABCs); CA, California; CT, Connecticut; +, positive; –, negative; COPD, chronic obstructive pulmonary disease; CHF, congestive heart failure.

contacts or the physicians about the use of prophylactic antibiotics. Thus, we were unable to consistently determine the number of household contacts who received prophylactic antibiotics specifically for the prevention of GAS disease from their physicians during the reference period. Another limitation of the study is related to the reasons why household contacts sought medical care. Although the chart abstraction form asked about chief complaints, it did not specifically ask if the contacts were asymptomatic and sought medical care simply because a family member had been ill with invasive GAS. We were therefore unable to consistently differentiate between household contacts who sought medical care for actual symptoms or illness from those who sought medical care simply because a family member had been ill from GAS.

Caution should be taken when defining the magnitude of increased risk for subsequent invasive GAS disease to household contacts compared with the risk for invasive GAS disease among the general population. The attack rates for confirmed and probable severe GAS disease in household contacts from this study are based on minuscule numbers (one and one, respectively), resulting in estimates with extremely wide confidence intervals. Even if the confirmed cases from this study and the Canadian study were combined, the point estimate would be based on five cases from 2,874 household contacts observed over several years of surveillance, and the confidence intervals would remain wide. Given that the combined population and duration of both studies are 22.8 million persons and 4.5 years, a well-designed prospective study of sufficient duration and size would be necessary to achieve a risk estimate with narrower confidence intervals and is likely not feasible.

Additionally, while both studies show an increase in risk for subsequent disease among household contacts, directly comparing the risk to the incidence of primary invasive disease is problematic (25). The attack rate of household contacts was determined during a 30-day period as opposed to a year because any risk for subsequent disease would likely be concentrated in the period shortly after the occurrence of the index case in the household. We think the data are best interpreted as additional evidence that household members are at higher risk for invasive GAS disease during the month following the index patient's illness than are others in the population but that the absolute risk for subsequent disease is low.

Because of the small numbers of case-pairs, predicting who is most likely to acquire a severe subsequent GAS infection is difficult based on either this study or the Canadian study. All five subsequent cases in the two reports occurred among adults who were immediate family members, and all five occurred within 3 weeks of the index patient's date of culture. Although we cannot predict who will acquire an invasive GAS infection from a household member, multiple published studies have identified those persons who are more likely to acquire sporadic invasive GAS infections that are unrelated to contact with infected persons and those who are more likely to die from an invasive infection. Groups at increased risk for sporadic disease include those who have recently been infected with varicella-zoster virus; have HIV infection, diabetes, cancer, or heart disease; are currently using high-dose steroids or intravenous drugs; or are Native American. Persons ≥65 years of age are more likely to die following an invasive GAS infection than other age groups (3,10,11,16).

This study provides important information for healthcare practitioners and public health personnel to help guide their responses to invasive GAS cases. The results of this study and the Canadian study, the potential impact of chemoprophylaxis, data on possible effectiveness of chemoprophylactic regimens, and the overall epidemiology of invasive GAS infections were recently reviewed by the Prevention of Invasive Group A Streptococcal Infections Working Group. The group concluded that although the risk for subsequent invasive GAS disease in household contacts is higher than the risk among the general population, routine administration of chemoprophylaxis to all household contacts of persons with invasive disease is not recommended given the infrequency of these infections and the lack of a known effective chemoprophylactic regimen (26). Clinicians and public health professionals should inform household members of persons with invasive GAS infections about the early clinical manifestations of pharyngeal and invasive GAS disease.

Ms. Robinson is an epidemiologist in the Office of Surveillance, Office of the Director, National Center for Infectious Diseases, Centers for Disease Control and Prevention. She received a bachelor of arts in psychology from Emory University and a master's degree in epidemiology from the Rollins School of Public Health at Emory University. Her research areas of interest include surveillance and infectious disease.

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Address for correspondence: Katherine Robinson, Office of Surveillance, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mailstop D59, 1600 Clifton Road NE, Atlanta, GA 30333, USA; fax: 912-635-3565; email: kad2@cdc.gov

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# Hepatitis E Virus Epidemiology in Industrialized Countries

Pilar Clemente-Casares,* Sonia Pina,* Maria Buti,† Rosend Jardi,† Margarita Martín,‡
Sílvia Bofill-Mas,* and Rosina Girones*

To determine the prevalence of Hepatitis E virus (HEV) in industrialized nations, we analyzed the excretion of HEV strains by the populations of Spain, France, Greece, Sweden, and the United States. Twenty of 46 (43.5%) urban sewage samples collected in Barcelona from 1994 to 2002 tested positive for HEV. We identified 15 HEV strains, which were similar to two HEV isolates previously described in Barcelona in clinical samples and to strains from diverse geographic HEV-nonendemic areas. We also identified two HEV strains in sewage samples from Washington, D.C., and Nancy, France; these samples were also positive for Hepatitis A virus. In addition, we studied the role of pigs as a reservoir for HEV and identified one new swine HEV strain. Our results suggest that HEV may be more prevalent than previously considered in industrialized countries and that variants of the virus circulate simultaneously in one region.

Hepatitis E virus (HEV) infection is a major cause of epidemic and acute sporadic hepatitis in many areas of Asia, Africa, and Mexico (1,2), where HEV is considered endemic. HEV is an RNA virus enterically transmitted with a single serotype, which affects mainly young adults. In countries where the virus is endemic, HEV is associated with >50% of sporadic acute hepatitis cases. The disease is self-limited but sometimes has severe complications and a high case-fatality rate, particularly in pregnant women (approximately 20%) (3). North America and Europe have traditionally been considered nonendemic for HEV; most HEV infections in those regions are considered to be imported, although seroprevalence ranges from 1% to 5% (4). In the last few years, some HEV strains associated with sporadic acute hepatitis have been isolated from human serum samples in North America (5) and Europe (i.e., Italy, Greece, Spain, and the United Kingdom) (6,7). Molecular analyses have shown that these strains form a group of HEV isolates that are genetically divergent compared with strains from HEV-endemic countries (8).

Evidence also exists that some animals can be reservoirs of HEV; for example, HEV infection has been demonstrated in swine (9). Swine and human HEV strains from a particular geographic region often appear to be closely related genetically (6,9–11). Transmission of HEV infection during outbreaks primarily occurs through contaminated water (12). Unlike other enterically transmitted infections, person-to-person transmission of HEV occurs infrequently (13).

We investigated the level of infection in regions where HEV is considered nonendemic by analyzing the excreted virus in the urban sewage of diverse geographic areas. The excretion and epidemiology of HEV was compared to the excretion and epidemiology of Hepatitis A virus (HAV) (14). We also analyzed swine fecal samples to identify evidence of HEV infection in pigs.

## Material and Methods

### Sewage Samples

Urban sewage samples were collected at the entry of a water treatment plant receiving 670,000 m³/day of waste products from Barcelona, Spain (population: approximately 1.8 million inhabitants). From the sewage network of this city, we collected 26 samples (taken every 2 weeks) from June 2000 to May 2001 and 8 samples (taken monthly) from June 2001 to January 2002. We also tested 12 sewage samples previously collected from the same area and stored at –80°C: 2 from 1994, 2 from 1995, 2 from 1996, 3 from 1997, 1 from 1998, and 2 from 1999. Each sample was collected in a sterile 500-mL polyethylene container, kept at 4°C for <8 h until viral particles were concentrated in phosphate-buffered saline (PBS, pH 7.3), and stored at –80°C.

We obtained urban sewage samples from other countries, as follows: five samples from Patras, Greece, in June–July 1999; five samples from Washington, D.C., United States, in December 1999; four samples from Nancy, France, in March 1998; and four samples from Umeå, Sweden, in September–October 1997. These samples were collected and shipped, frozen, to Spain where the viral particles were concentrated in PBS and stored at –80°C.

### Human Serum Samples

We contacted 13 patients seen in the emergency room of the Hospital General Valle Hebron, Barcelona, Spain, for acute hepatitis over the last 12 years who had tested positive for immunoglobulin (Ig) G anti-HEV. A follow-up serum sample was collected in order to evaluate the durability of antibody response. These samples were stored at –80°C.

*University of Barcelona, Barcelona, Spain; †Hospital General Universitario Valle Hebron, Barcelona, Spain; and ‡Universitat Autònoma de Barcelona, Barcelona, Spain

## Animal Samples

Seventy-three serum samples were obtained from healthy pigs in three commercial herds in Catalonia, Spain: 43 weaned pigs (3–9 weeks old), 10 fattening pigs (10–22 weeks old), 8 gilts (young sows), and 12 sows. Blood samples were taken aseptically from the vena cava or jugular vein, and the serum obtained by centrifugation was stored at –80°C until tested.

Thirty fecal samples from pigs of various ages were collected from the same three herds. Individual samples were taken and later pooled with samples from 3–5 animals in the same age group. Samples from the "fattening" pigs were collected from different places in their pens, each one containing 10–15 animals.

## Serologic Tests of Human Serum Samples

Commercially available enzyme immunoassays (Abbott Laboratories, Abbott Park, IL) were used for the detection of hepatitis B surface antigen, IgM anti–hepatitis B core, IgM anti-hepatitis C virus (HCV), anti-HAV, and IgG anti-HEV. HCV-RNA was analyzed by polymerase chain reaction (PCR) (Amplicor HCV, monitor test version 2.0; Hoffman-La Roche Inc., Nutley, NJ).

## Serologic Tests of Swine Serum Samples

A commercially available enzyme immunoassay (Abbott Laboratories) was used, with some modifications to detect IgG anti-HEV in pigs. Hyperimmune serum from a pig experimentally inoculated with the porcine-HEV strain and preimmune serum from a noninoculated pig and a Rhesus Monkey (*Macaca mulatta*) (serum donated by S.U. Emerson and R.H. Purcell) were used as positive and negative controls, respectively.

## Control Viruses

Fecal suspensions obtained from Rhesus Monkeys infected with HEV Barcelona (BCN) strain (10% in PBS, pH 7.3) were used as positive control for the PCR analysis. The strain that infected the monkeys was isolated from sewage in Barcelona and is genetically similar to Indian strains (15). Viral suspensions were stored at –80°C.

## Concentration of Viral Particles from Sewage Samples

Recovery of viral particles was carried out as described previously (16,17). Briefly, a 40-mL sewage sample was ultracentrifuged ($110,000 \times g$ for 1 h at 4°C) to pellet all viral particles together with any suspended material. The sediment was eluted by using 4 mL 0.25 N glycine buffer, pH 9.5, and the suspended solids were separated by centrifugation at $12,000 \times g$ for 15 min. Viruses were finally pelleted by using ultracentrifugation ($110,000 \times g$ for 1 h at 4°C), resuspended in 0.1 mL PBS, and stored at –80°C.

## Concentration of Viral Particles from Swine Feces Samples

A protocol similar to the one used for sewage samples was carried out to concentrate viral particles from swine feces samples; 1 g of a pool of feces was eluted in 4 mL 0.25 N glycine

buffer, pH 9.5, and centrifuged ($10,000 \times g$ for 15 min) to separate suspended solids. The supernatant was finally ultracentrifuged ($110,000 \times g$ for 1 h at 4°C), and viruses were resuspended in 0.1 mL PBS and stored at –80°C.

## Nucleic Acid Extraction

Viral nucleic acids from viral particles were extracted as described previously (18) after we selected the procedure that is more likely to eliminate potential inhibitors of reverse transcription (RT)–PCR. The method is based on the use of guanidinium isothiocyanate and adsorption of the nucleic acids to silica particles. Briefly, 50 µL of viral concentrate was added to a mixture of 50 µL of silica particle suspension and 900 µL of lysis buffer. The mixture was incubated at room temperature for 10 min and washed twice in 1 mL of washing buffer (12 g of guanidine thiocyanate in 10 mL of Tris-EDTA), twice more in 70% ethanol, and once in acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 50 µL of elution buffer (49.4 µL of dithiothreitol [DTT] and 0.6 µL of RNase inhibitor [Applied Biosystems, Foster City, CA]). The extracted nucleic acids were then used for cDNA synthesis and amplification of the HEV and HAV genomes.

## Enzymatic Amplification

To detect viral RNA, we used a seminested RT-PCR with degenerated primers as described (19). Five µL of the extracted nucleic acids and a 10-fold dilution (corresponding to 5 µL and 0.5 µL of serum, 50 mg and 5 mg of feces, and 2 mL and 0.2 mL of sewage) were analyzed by RT-PCR, plus 1.5 mM of $MgCl_2$, PCR Gold Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) (Applied Biosystems), 0.01 M DTT, 10 nmol of each dNTP, and 25 pmol of the reverse primer (HEVORF2con-a1; nt 6454–6479 in HEV United States [US1] strain) in a total volume of 10 µL. The mixture was incubated at 95°C for 5 min before the addition of 10 U of ribonuclease inhibitor (Applied Biosystems) and 50 U of RT mouse mammary leukemia virus (Applied Biosystems). After 30 min at 42°C, the mixture was heated again at 95°C for 5 min.

For a typical one-step reaction, we used 10 µL of the cDNA solution. Amplification was carried out in a 50-µL reaction mixture containing PCR Gold Buffer (Applied Biosystems), 1.2 mM $MgCl_2$, 2 U of Ampli Taq Gold (Applied Biosystems) 25 pmol of the forward primer (HEVORF2con-s1; nt 6283–6306 in the HEV US1 strain). The first cycle of denaturation was carried out for 5 min at 95°C, followed by 35 cycles at 94°C for 60 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. All amplifications were completed with a 10-min, 72°C extension period. For a second PCR amplification cycle, we added 1 µL of the first-round product to a new batch of 50-µL reaction mixture containing 25 pmol of each primer (HEVORF2con-a1 and HEVORF2con-s2; nt 6332–6353 in US1 strain). This second PCR was performed under the same conditions. The products were analyzed by agarose gel electrophoresis with ethidium bromide stain. A region of 148 nucleotides within open reading frame (ORF) 2 of the HEV

genome was amplified. Also, assays for the amplification of a region of 287 nucleotides within ORF1 were carried out. Primers used were HEVORF1con-s1 (nt 2–25), HEVORF1con-s2 (nt 50–70), HEVORF1con-a1 (nt 397–419), and HEVORF1con-a2 (nt 313–336). The primer positions are relative to HEV US1 strain. Nested RT-PCR was carried out for detecting HAV genome in sewage samples as described (14), amplifying fragments within the 5´ nontranslated region (NTR) region and the VP1/A2 junction region.

### Quality Control of the Amplification Method

Standard precautions were used for all procedures to reduce the possibility of sample contamination by amplified DNA molecules. A negative control was added every two samples. Virus-positive amplification results were confirmed by sequencing.

### Sequencing and Analysis of the Viral Genome

The amplicons obtained after the nested PCR were purified by using the QIAquick PCR purification Kit (QIAGEN GmbH, Inc., Hilden, Germany), following the manufacturer's instructions. Both strands of the purified DNA amplicons were sequenced with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq DNA polymerase FS (Applied Biosystems), following the manufacturer's instructions. We checked results using the ABI PRISM 3700 DNA analyzer (Applied Biosystems). We compared the sequences with those present in GenBank and the European Molecular Biology Library by using the basic BLAST program of the National Center for Biotechnology Information (available from: URL: http://www.ncbi.nlm.nih.gov/BLAST/). GenBank accession numbers of the HEV strains sequences characterized previously and used for phylogenetic studies are listed in Table 1.

Alignments of the sequences were carried out by using the ClustalX 1.8 program (available from: ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Phylograms were generated by the UPGMA algorithm using the NEIGHBOR program. The robustness of the grouping was determined by bootstrap resampling of the multiple sequence alignments (1,000 sets) with the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE. The output graphics of the trees were created with the TREEVIEW package, version 1.5 (20).

## Results

### Sewage Samples

Forty-six sewage samples from Barcelona were collected from 1994 to 2002. Of the samples collected before 2001, a total of 4 (14.8%) of 27 tested positive for HEV RNA; this proportion increased to 84.2% (16/19) among the samples collected from 2001 to 2002 (Table 2). After performing sequence analysis of the amplified region within ORF2, we identified 15 new HEV isolates (BCN2–BCN16) (GenBank accession nos. in Table 1).

Table 1. Nucleotide sequence accession numbers for Hepatitis E virus strains

| Origin | Abbreviation | GenBank accession no. |
|---|---|---|
| **Described in previous studies** | | |
| Barcelona | BCN | AF058684 |
| Greece | G1 | AF110391 |
| | G2 | AF110392 |
| Italy | It | AF110390 |
| Austria | Au | AF279123 |
| United States | US1 | AF060668 |
| | US2 | AF060669 |
| | Sw[a] | AF082843 |
| Mexico | M | M74506 |
| Pakistan | P | M80581 |
| Burma | B | M73218 |
| China | C1 | D11092 |
| | C2 | AJ272108 |
| | C3 | AF082094 |
| India | I | X98292 |
| **Reported in this study** | | |
| Barcelona | BCN2 | AF491004 |
| | BCN3 | AF490985 |
| | BCN4 | AF491003 |
| | BCN5–BCN16 | AF490986–AF490997 |
| Washington, D.C., United States | W1 | AF490998 |
| Nancy, France | N1 | AF490999 |
| Barcelona, Spain[b] | VH1 | AF491000 |
| | VH2 | AF491001 |
| Catalonia, Spain[a] | Por1 | AF491002 |

[a]Originated from swine.
[b]Strains reported previously (15), but their sequences within open reading frame 2 used for phylogenetic analysis in this study are new.

HAV RNA was detected in 21 (77.8%) of 27 sewage samples that were collected in Barcelona from 1994 to 2000 and tested for the presence of HAV. Previous studies in the same area showed 31 (57.4%) of 54 sewage samples positive for HAV from 1994 to 2000 (14).

We also tested for the presence of HEV RNA in sewage samples from other countries where HEV was previously considered nonendemic. One of five samples from Washington, D.C., and one of four samples tested from Nancy, France, were positive, showing two new HEV strains (W1 and N1). HEV RNA was not detected in any of the four samples from Umeå, Sweden, or the five samples from Patras, Greece. HAV RNA was detected in all four countries: All U.S. sewage samples, three of four samples from France, one of five samples from Greece, and one of four from Sweden tested positive for HAV.

### Human Serum Samples

Follow-up serum samples from 13 seropositive acute hepatitis patients from Barcelona were analyzed to evaluate the durability of antibody response. None of these samples showed the RNA of the virus. IgG anti-HEV was undetectable in 7 (53.8%) of 13 of these follow-up samples. Seven of eight seropositive patients were found to be seronegative 2 to 12 years after the initial test.

Table 2. Summary of results of HEV and HAV found in urban sewage samples

| Site | Period of sampling | Positive HEV samples/total analyzed | Positive HAV samples/total analyzed |
|---|---|---|---|
| Barcelona, Spain | October–November 1994 | 0/2 | 1/2 |
| | May–June 1995 | 0/2 | 1/2 |
| | February–April 1996 | 1/2 | 1/2 |
| | September–October 1997 | 0/3 | 2/3 |
| | January 1998 | 0/1 | 1/1 |
| | March–April 1999 | 1/2 | 2/2 |
| | June–December 2000 | 2/15 | 13/15 |
| | January 2001–January 2002 | 16/19 | NT |
| Total (for Barcelona) | | 20/46 (43.5%)[b] | 21/27 (77.8%)[b] |
| Nancy, France | March 1998 | 1/4 | 3/4 |
| Umeå, Sweden | September–October 1997 | 0/4 | 1/4 |
| Patras, Greece | June–July 1999 | 0/5 | 1/4 |
| Washington, D.C., United States | December 1999 | 1/5 | 5/5 |

[a]HEV, Hepatitis E virus; HAV, Hepatitis A virus; NT, not tested.
[b]% of positive samples.

## Animal Samples

Swine serum and fecal samples from pigs in three different herds in Catalonia, Spain, were tested. A total of 10 (13.7%) of 73 serum samples were positive for anti-HEV IgG antibodies (all were obtained from fattening pigs or the oldest pigs from the same herd). A total of 55 serum samples from pigs of different ages were taken at this farm. The distribution of seropositive animals was as follows: 4 (40.0%) of 10 samples from fattening pigs, 2 (40.0%) of 5 from gilts, 1 (33.3%) of 3 from primiparous sows, and 3 (42.8%) of 7 from multiparous sows. Pigs from the other two farms were seronegative for HEV. None of the serum samples were positive for HEV by RT-PCR.

The HEV genome was amplified by RT-PCR in 6 of 12 fecal samples collected in the herd with seropositive animals. We detected HEV in one of the two pools from pigs 3 weeks old, in one of two pools from pigs 5 weeks old, in two of two pools from pigs 8 weeks old, in one of two pools from fattening pigs, and in the pool of feces from primiparous sows. After sequence analysis, we identified one new swine HEV strain (Por1).

## Sequence Analysis of the HEV RNA Genomes

Phylogenetic analysis showed that all the sequences detected were grouped with strains isolated in countries where HEV was considered nonendemic (Figure 1). The nucleotide sequence alignment of the detected strains with other HEV strains is shown in Figure 2. BCN2–9, BCN11–BCN14, and BCN16 shared a 91.0% to 99.2% similarity. They were closely related to VH1 (94.3% to 98.4% identity) and VH2 (91.0% to 95.1%), two strains isolated from clinical samples in the same area (Barcelona), previously described (15), and G1 (92.7% to 96.7%), an isolate from Greece. Two isolates (BCN10 and BCN15) showed substantially different sequences, sharing 86.5% to 89.2% and 87.2% to 91.9% similarity with the rest of strains detected in Barcelona. A previous study (15) in the same area with specific primers for HEV strains from areas where the virus is endemic identified one isolate (BCN), which was closely related to Indian strains. Nucleotide sequence identities among this isolate and the new ones from Barcelona ranged from 79.7% to 83.7%.

The HEV strain identified in sewage from Washington (W1) was closely related to other isolates from the United States, sharing 91.0% to 91.9% identity with US1 and US2 strains obtained from human serum samples, and 98.4% with a swine strain (Sw) also isolated in the United States. BCN15, from Barcelona, was also similar to those isolates from the United States, showing 89.4% to 91.9% nucleotide sequence identity. The sample from Nancy (N1) was highly similar (92.7%) to VH1. All HAV strains were genotype IA, except the Swedish isolate (IB).

The nucleotide sequences of the isolates from swine were identical and, when compared with other isolates, the Por1 strain exhibited a similarity of 92.7% with G2 and 87.0% to
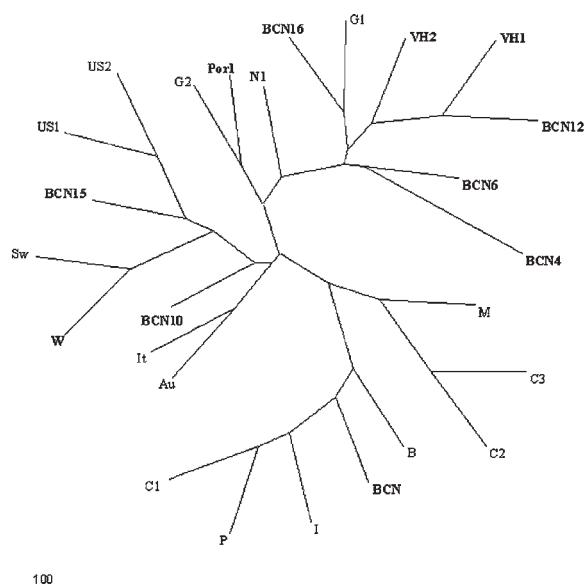


Figure 1. Unrooted phylogenetic tree showing the relationship over a 123-bp fragment within open reading frame 2 between representative Hepatitis E virus strains reported in this study and other isolates from genotype I (C1, China; P, Pakistan; I, India; BCN, Barcelona, Spain; and B, Burma), genotype II (M, Mexico), genotype III (US1 and US2, United States; Sw, swine, United States; G1 and G2, Greece; It, Italy; and Au, Austria), and genotype IV (C2 and C3, China). Strains from Barcelona, Spain, Washington, D.C., United States, and Nancy, France, are in bold.
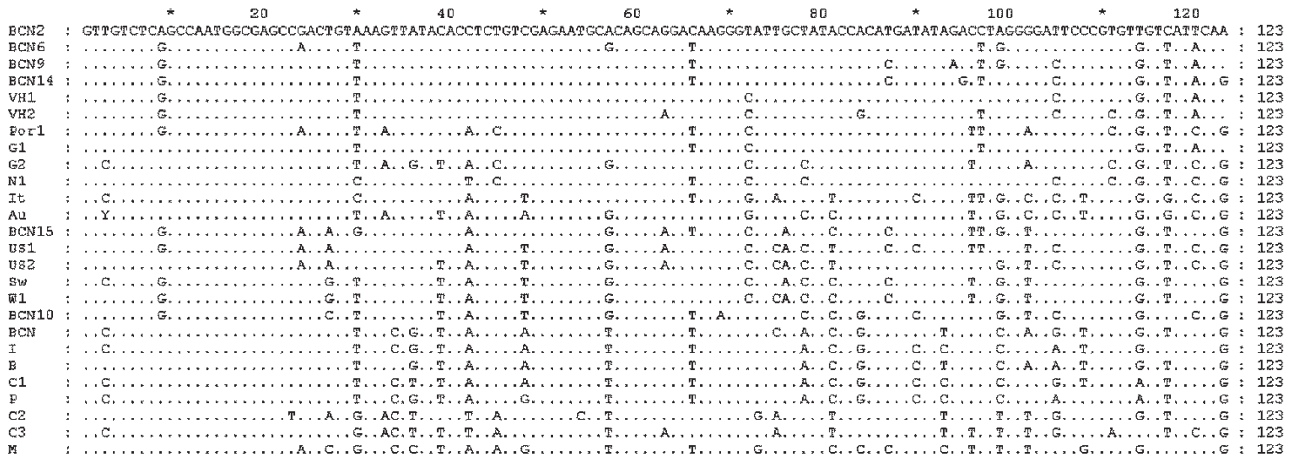
Figure 2. Nucleotide alignment of the amplified fragment within ORF2 from some representative isolates in this study with other Hepatitis E virus strains. Dots indicate sequence identities.

91.0% with isolates from sewage in Barcelona. The results of translating nucleotide sequences into amino acid sequences showed that the substitutions in strains detected in Barcelona (including the one from pigs) were mainly located in the third codon position and were conservative (Figure 3). Two strains (BCN9 and BCN14) showed two nonconservative substitutions in the same position.

## Discussion

In previous studies, investigating the viruses present in a population's sewage has produced reliable information about the strains infecting that population; this type of analysis detects the viruses that cause clinical and subclinical infections in the population (14). In our study, we showed the presence of HEV strains during a period of several years in raw urban sewage from an area previously considered nonendemic for the virus. We also demonstrated the presence of HEV in other industrialized countries by testing urban sewage samples from diverse geographic areas. We identified 17 new HEV strains from sewage samples in Barcelona, Spain (15 isolates); Washington, D.C., United States (1 isolate); and Nancy, France (1 isolate); these locations also produced a high percentage of HAV-positive sewage samples.

The amplified region of the viral genomes detected in this study was sequenced; the diversity we observed indicates the absence of a unique HEV outbreak in the population and shows that a diverse number of strains are simultaneously circulating. Only the viral genomes from the samples showing the higher viral concentration (Por1 and W1) could be sequenced in ORF1 by using the described primers. Y. Wang et al. (21) noted that negative results were observed in serum samples positive in the ORF2 when using these ORF1 primers, suggesting a lower level of efficiency in the amplification reactions compared with the ORF2 primers applied in the study. The phylogenetic analysis of short sequences has previously produced trees similar in structure to those observed with longer sequences (21,22). The strains we describe may be most closely related to genotype III,

one of the four genotypes described by G.G. Schlauder and I.K. Mushahwar (8). Larger fragments should be sequenced in order to confirm these results.

The prevalence of HAV infection in Catalonia, where most of the sewage samples were collected, is 67.8% (23); in this area, HAV is considered to be of intermediate endemicity. In the same area of Barcelona, 57.4% of the sewage samples collected from 1994 to 2000 showed HAV; these HAV strains were found to be closely related to the HAV strains identified in the clinical samples for the same period (14).

HEV can be detected in the stool of most patients infected with HEV, with duration of fecal shedding generally limited to 9–12 days (24). If we compare the transmission of HEV and HAV in this region, since the time after infection that HEV is
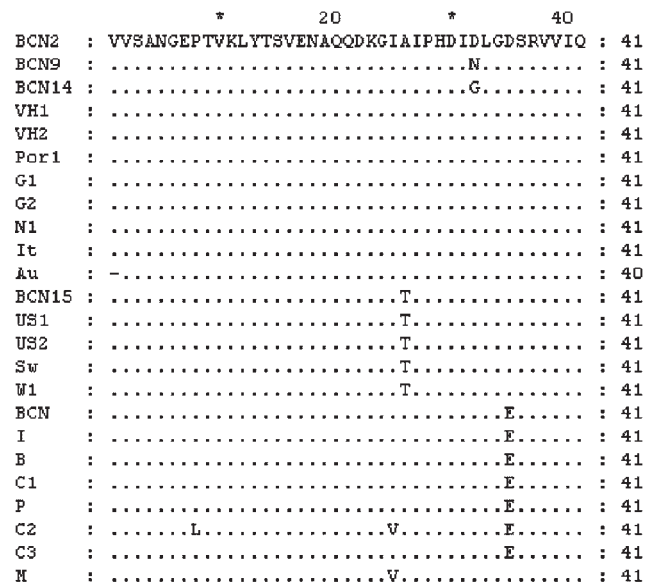


Figure 3. Amino acid alignment from representative isolates in this study with other Hepatitis E virus strains. Amino acid sequences from BCN3–BCN8, BCN10–BCN13, and BCN16 are identical to BCN2. Dots indicate sequence identities.

excreted does not appear to be longer than the time after infection that HAV is excreted, we find that HEV infections may be more frequent than previously considered. That 84.2% of sewage samples collected during 2001 and 2002 were HEV positive suggests that the prevalence of HEV infections may be underestimated.

In our study we also analyzed samples from three commercial pig farms; only one of these farms had pigs positive for HEV. However, from that one farm, antibodies and virus were detected in animals of different ages, suggesting that HEV is widespread among animals in infected herds. Our serologic results showed that although only 13.7% of samples were positive, all of them were from animals >10 weeks (fattening pigs and sows). Since that farm worked with a farrow-to-finish system, these results may indicate a slow diffusion of infection within the farm. The fact that adult sows had positive serologic and virologic test results suggests their possible role as reservoirs on the farm. The swine HEV strain identified in these animals has been found to be related to the human strains detected (87.0% to 91.0%). Previous studies also reported that swine HEV isolates identified in the United States (9), Taiwan (11), and Japan (10) were closely related to human HEV isolates obtained from the same geographic areas. In fact, swine HEV isolates seem to be more similar to human HEV isolates from the same area than they are to swine HEV isolates from different geographic areas (25). Swine HEV isolates identified in Canada and the Netherlands have been clustered with previously described European and American human or swine isolates (26,27). Finally, veterinarians and persons working with pigs have been shown to at a greater risk for HEV infection. Meng et al. (28) reported that swine veterinarians were one and a half times more likely to seroconvert to HEV infection, although clearly multiple sources of exposure can exist.

Most existing assays for antibody to HEV are enzyme immunoassays that use recombinant-expressed proteins or synthetic peptides representing antigenic domains from ORF 2 and ORF 3, commonly from strains of at least two geographically distinct HEV strains. The HEV strains used in these tests are representative of those from countries with endemic HEV and show some differences from recently identified strains in the sequence of amino acids of several major epitopes as the region near the carboxyl ends of ORF 2 and ORF 3. Some differences are shown in amino acid sequences of strains from areas with nonendemic and endemic virus in the amplified region, which is located within a strongly reactive epitope (29). This diversity could be producing a lower level of sensitivity in the serologic assays for these infections. A recent study from the Hepatitis E Virus Antibody Serum Panel Evaluation Group concluded that discrepant results among blood donor serum samples show that anti-HEV seroprevalence data in countries with nonendemic HEV may be unreliable and should be interpreted with caution (30). Our results also support this conclusion. As some authors have suggested (31), we show that IgG antibodies a few years after the initial test are reduced to undetectable levels in 53.8% of cases. In some cases, antibody levels are reduced very early, after only 3–4 months, which makes the diagnosis of HEV infection extremely difficult. Several authors have suggested that some persons may not produce a detectable antibody response at all (24). Balayan (3) also considered the prevalence of anti-HEV in areas endemic for HEV to be much lower than expected, with a rate of 2.8% to 20.2% in areas having a high proportion of HAV-seropositive persons. In highly industrialized countries, anti-HEV has been regularly found at a rate not exceeding 5%; in Spain, anti-HEV was found in 1% to 3% of blood donors (32).

Specific information on the pathogenicity of the HEV strains detected in Europe and the United States is not available; these data are required for evaluating the potential risk associated with the infection. Using adequate specific tests must be considered for the routine diagnosis of HEV infections in patients with acute viral hepatitis not related to HAV in industrialized countries, especially in asymptomatic patients with unexplained elevated aminotransferase levels and in pregnant women. Our data suggest that HEV strains are more widespread in the human population than previously thought and endemic HEV infections are likely present in Europe and the United States. More studies are warranted to characterize the HEV infections detected and evaluate the sanitary risk that the excreted HEV represents for humans.

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Ms. Clemente-Casares is a doctoral student at the University of Barcelona. She is working on the detection of human virus by molecular methods in clinical and environmental samples focused on the study of the epidemiology of Hepatitis E and Hepatitis A viruses.

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Address for correspondence: Rosina Girones, Department of Microbiology, Faculty of Biology, University of Barcelona, Avd. Diagonal 645, 08028 Barcelona, Spain; fax: 34-93-4110592; email: rgirones@ub.edu

www.cdc.gov/eid

# Infectious Pancreatic Necrosis Virus in Scottish Atlantic Salmon Farms, 1996–2001

Alexander G. Murray,*† Corina D. Busby,* and David W. Bruno*

The rapid growth of aquaculture has provided opportunities for the emergence of diseases. Programs designed to monitor these pathogens are useful for analysis of regional variation and trends, provided methods are standardized. Data from an official monitoring program were used to analyze the emergence of infectious pancreatic necrosis virus in Scottish salmon farms from 1996 to 2001. An annual increase in the prevalence of this virus was found in saltwater (10%) and freshwater sites (2% to 3%), with a much faster increase (6.5%) in Shetland's freshwater sites. No significant increase in the virus was detected in the marine farms of southern mainland Scotland. However, the virus had become very prevalent at marine sites and was almost ubiquitous in Shetland by 2001. The prevalence of this virus at marine sites may be underestimated. Because several diseases have emerged or are emerging in fish farming, aquaculture surveillance programs represent a rich potential source of data on emerging diseases.

The growth of the aquaculture industry provides a rich environment in which to study emergence of pathogens (1,2). New fish species are being cultivated, and production has risen rapidly. For example, from 1996 to 2001, Scottish farmed salmon production doubled to 158,000 metric tons (3). Shipping is extensive, and stock movement between farms is substantial (4). Thus, both host populations and contact opportunities are high, the ideal theoretical conditions for disease transmission (5). Indeed, an array of pathogens that affect farmed salmon have emerged (6).

Because of the disease threat, official (6,7) and industry (8) surveillance for pathogens is extensive. This surveillance is designed to find pathogens, and thus may bias estimates of true pathogen prevalence. Sampling may be biased to farms perceived to be at risk or to specific times of year. The number of fish in a sample may vary. Similarly, sampling methods have detection limits, which may result in substantial numbers of asymptomatic infections being undetected (9,10). Nevertheless, a continuous centrally organized national survey exists for pathogens in an environment where pathogens are emerging. This survey has the potential to throw detailed light on patterns of pathogen emergence, even if absolute prevalence may be biased.

*Fisheries Research Services Marine Laboratory, Victoria Road, Aberdeen, Scotland; and †Department of Zoology, University of Aberdeen, Aberdeen, Scotland

Infectious pancreatic necrosis (IPN) is considered the most serious viral disease in terms of its impact on Atlantic salmon (*Salmo salar*) production in the European Union (11); the disease occurs on four continents (7). The problem is increasing as the disease continues to emerge (12). IPN is caused by the *Infectious pancreatic necrosis virus* (IPNV; genus *Aquabirnavirus*) (13,14). Presence of IPNV is a necessary, but not sufficient, condition for IPN; disease also depends upon viral strain (15), environment (12), and age of the fish (12,13). In nondiseased fish, IPNV may be present in small quantities (13,16); virus in such carrier fish may be difficult to detect, which leads to underreporting (9,10).

IPN has been known for decades. It was first described in North America in freshwater trout in the 1950s (17), although indications of the disease were reported in 1940 (16). IPN was first reported in the United Kingdom in 1971 (18) in rainbow trout, *Oncorhynchus mykiss,* a North American species. Early reports of IPN in salmonids were associated with deaths of fry (recently hatched fishes) in freshwater hatcheries (13), but since the 1980s, deaths have also been increasingly reported among smolts (2-year-old salmon) shortly after transfer to seawater (15,12). IPNV-positive salmon smolts are estimated to have a mortality rate five times higher than IPNV-negative smolts (14). IPN is also associated with less easily quantified losses such as loss of appetite and therefore of production (19).

## Methods

Fisheries Research Services (FRS) fisheries inspectors have sampled fish from throughout Scotland for pathogens, including IPNV, for many years. The inspectorate is based in Aberdeen (Figure 1), distant from salmon farming areas on the west coast and Northern Isles (3). This central organization is important for the sampling structure because the same persons collected samples from different regions using standard operating procedures, and a single laboratory then processed these samples.

The IPNV diagnosis used cell culture on Chinook salmon embryo cell lines in plates containing 24 wells (7). Virus identification was undertaken with enzyme-linked immunosorbent assay (7). The standard sample from 30 fish was divided into six pools, each containing material from five kidneys; occasionally 30 pools from individual kidneys were sampled. However, some samples did not contain 30 fish, depending
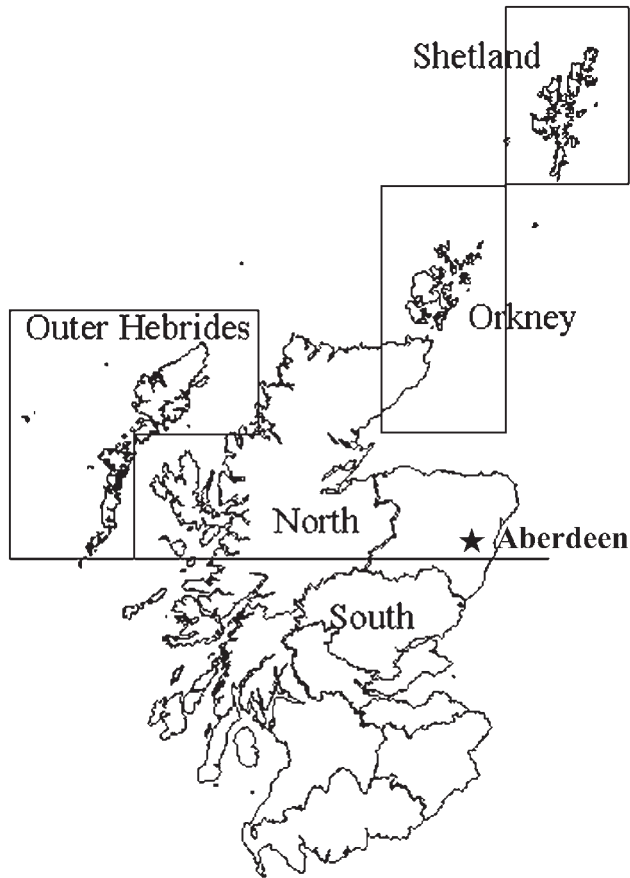
Figure 1. Salmon production regions in Scotland, including the city of Aberdeen, the site of the Fisheries Research Services Marine Laboratory, where fish health inspectors and virologists are based.

upon opportunity, and varied from 1 to 150 fish kidneys; pool size also varied. Any positive pool generated a positive result at the sample level. Because samples with smaller numbers of fish are less likely to contain virus than are large ones (20), IPNV may be missed in small samples. In addition, limits of test sensitivity mean that fish with low IPNV titers may escape detection (9,10), although if samples are large enough to contain several infected fish, test sensitivity is likely to be less important in detecting IPNV at the sample level (20). Small samples from biased locations and test detection limits mean that errors may exist in the absolute prevalence estimates, but with standard methods and centralized personnel, trends and relative regional prevalence can be determined.

FRS scientists have been testing for IPNV since the 1970s (18); standard methods approved by the Office International des Epizooties (7) were used through the 1990s. In freshwater, sonication has been applied to tissue homogenate since 1993 to disrupt cells and release virus; in saltwater, this process was not begun until 2001. Thus, sensitivity of detection may have been slightly improved for marine samples in the last year and was generally better in freshwater.

Data available for 1996–2001 have been used to find underlying patterns in the distribution of prevalence of IPNV in Scottish salmon farms. Fish were sampled at all stages of the

production cycle, although many more samples were collected in summer than in winter, leading to potential biases. Also, inspectors preferentially take moribund fish because these are of most concern and could indicate a disease outbreak. Most sites were only sampled at most once in a given year, but a minority (8.5% in 1996, 12.3% in 1997, 7.9% in 1998, 15% in 1999, 26% in 2000, and 31% in 2001) were sampled on two or more occasions in a single year. All farms are sampled at least once every 2 years. Data are insufficient to systematically analyze variation at sites.

The mean prevalence of IPNV was calculated for classes of samples, e.g., marine salmon farms in Shetland in 1996. (Prevalence was the proportion of samples that were IPNV positive for the class of site examined.) The use of prevalence removed the bias associated with different sampling frequencies. Confidence intervals were determined from the probability values for which distribution intercepts with observations, for the number of observations available. For a more formal test of when regional means deviated significantly from the overall mean, we used the $\chi^2$ test, because the data were binomially distributed. Site samples were not fully independent, but most sites were not sampled more than once in a year and thus were semi-independent. Clumping of samples could also have occurred for sites within hydrodynamically well-mixed environments (e.g., farms in the same sea loch).

Regional patterns exist for IPN; Shetland has a particularly serious problem (16). Regional variation in IPN-induced deaths is also apparent in Norway (12). Regional prevalence patterns are therefore examined by separating out salmon production sites into regions based on a United Kingdom ordnance survey 100-km² grid. The regions identified are Shetland, Orkney, the Outer Hebrides, and mainland Scotland. Mainland Scotland was divided into northern and southern regions by using the 800-km north line of the United Kingdom ordnance survey's national grid system (Figure 1). The interannual changes in IPNV prevalence have been examined at salmon production sites within the regions identified above.

## Multilevel Model

Both space and time play roles in determining variance in the observations. Simple multilevel models of the variance in the observations allow determination of the relative contributions of these components. These models, based on normal or binomial assumptions, were constructed by using the package MLwiN (21). The model was constructed with binomial variance distributed according to a logistic function IPNV = [1 + exp(−β)]⁻¹. This model determined a mean β = −1.138 (±0.517), which gave a mean prevalence of 0.242 (24%). The logistic function was used because it varies from 0 to 1 and therefore covers the range of situations when IPNV is absent to situations when it is present in all samples within a group. The model was then used to determine the contributions of the different levels in the model to the variance about this mean.

The highest level of the model is the split between saltwater and freshwater environments. These salmon production

sites are fundamentally different in nature (3), with different age classes of fish and routes of infection. Inclusion does add uncertainty to the model's output because environment has only two classes. However, because saltwater and freshwater sites are related at other levels in different ways, this split in variance is explicitly modeled, rather than added as a fixed effect. The second level is the region; at this level, different climates, environments, and local organizations (e.g., the Orkney Fish Farmers Association) exist. The third level at which variance is resolved is that of year: because IPNV's prevalence is increasing, prevalence changes over time. Seasonal data are also included because most IPNV cases occur in summer. Seasonal effect was analyzed by using three seasons of 4 months' duration (January–April, May–August, and September–December) because individual months in winter are often poorly sampled.

## Results

Mean IPNV prevalence was determined by region, and a $\chi^2$ test of deviation from the overall Scottish mean was conducted (Table 1). High IPNV levels stood out in Shetland for both freshwater and seawater sites. Northern mainland Scotland and the Outer Hebrides showed a particularly low relative prevalence of IPNV in the marine sites and marginally significant low prevalence in freshwater. Orkney also had a low prevalence of IPNV in freshwater; however, because we obtained fewer data from Orkney, this finding was not significant.

In marine sites, IPNV prevalence showed different temporal patterns in different regions (Figure 2). In most areas, IPNV prevalence appeared to have increased from 1996 to 2001 at approximately 10% per year (Table 2); the trends in these regions were not significantly different from each other or from the national mean. Analysis methods changed slightly in 2001; however, the trend of increasing prevalence was apparent throughout the entire 1996–2001 period. In southern mainland Scotland, IPNV prevalence was not statistically significant. If anything, it declined, although variation occurred from year to year. Linear increase in IPNV prevalence in Shetland became difficult to sustain once prevalence exceeded 80% in 1999, so the increase reached a saturation point (Figure 2).

In freshwater production sites, interannual variation in IPNV prevalence increased approximately 2% to 3% per year (Figure 3, Table 2). However, in Shetland, the increase was faster at 6.5% year, which is significantly different from other
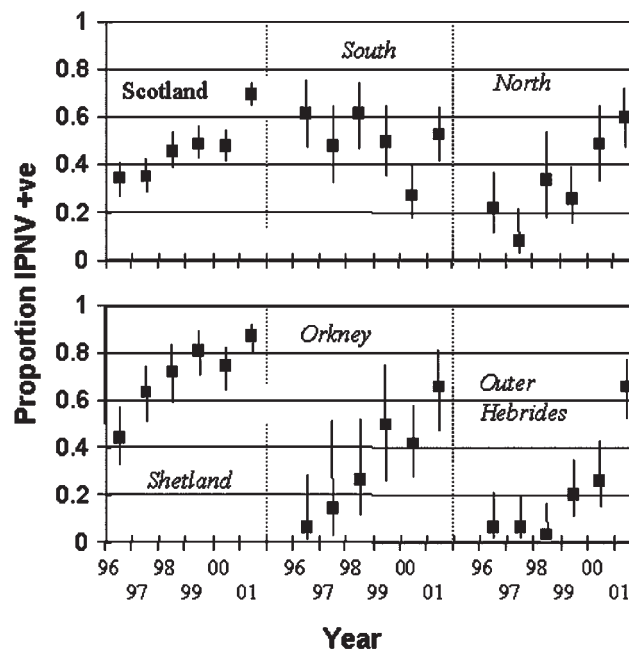


Figure 2. Prevalence of infectious pancreatic necrosis virus (IPNV) in Scottish marine salmon production sites by region and year. The regions are northern mainland Scotland, southern mainland Scotland, Shetland, Orkney, and the Outer Hebrides. Bars show 95% confidence intervals.

regressions at 90% confidence. The analysis used has not changed for freshwater since 1993. In northern Scotland, no significant increase has occurred, although since 1999, prevalence has increased. As noted earlier, prevalence of IPNV in Shetland in freshwater is higher than in other areas and is increasing more rapidly.

Differences between regions have decreased in recent years in spite of increasing prevalence (Figure 4). The regions are becoming less distinct with respect to IPNV prevalence as the IPNV-positive regions converge.

The multilevel model has been used to determine how much different components contribute to the variation in model results from the overall mean of 24% (Table 3). The contribution is distributed, as expected from the observations described earlier. Most variance is accounted for at level 2, between regions. A substantial amount of variance is at the top level between saltwater and freshwater environments, but, because only two categories exist, the degree of this variance is uncertain. Since slightly more sensitive methods were used to sample IPNV in freshwater than in saltwater, this difference may be underestimated. A similar amount of variance is accounted for at the interannual level but with less uncertainty. Very little variation is due to season.

## Discussion

Our analysis systematically shows that variation exists in the distribution of IPNV in Scotland and allows a detailed picture of changes in distribution to be derived. The emergence of IPNV has been documented, with IPNV prevalence increasing

Table 1. Mean prevalence of infectious pancreatic necrosis virus by region for all 1996–2001 data, and $\chi^2$ probability that prevalence is significantly different from the all-Scotland mean

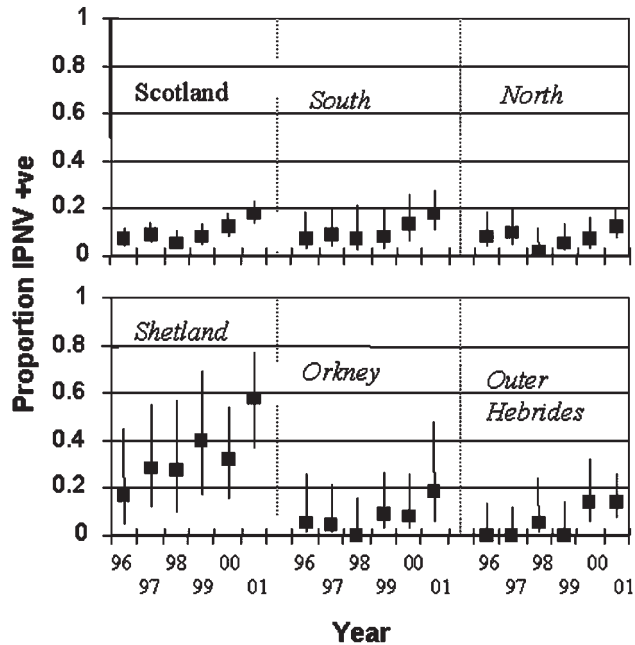| Region | Saltwater | | Freshwater | |
|---|---|---|---|---|
| | % Prevalence | $\chi^2$ p | % Prevalence | $\chi^2$ p |
| All Scotland | 49.6 | N/A | 10.6 | N/A |
| North | 41.1 | 0.001 | 5.5 | 0.1 |
| South | 55.5 | – | 11.1 | – |
| Shetland | 77.0 | 0.001 | 35.3 | 0.001 |
| Orkney | 48.5 | – | 6.7 | – |
| Outer Hebrides | 31.3 | 0.001 | 6.7 | 0.1 |

Figure 3. Prevalence of infectious pancreatic necrosis virus (IPNV) in Scottish freshwater salmon production sites by region and year. The regions are northern mainland Scotland, southern mainland Scotland, Shetland, Orkney, and the Outer Hebrides. Bars show 95% confidence intervals.

Table 2. Trends in annual average prevalence of infectious pancreatic necrosis virus for all Scotland and by regions with regression 90% confidence intervals, probability, and $r^{2a}$

| Region | % Annual increase | 90% CI | p | $r^2$ |
|---|---|---|---|---|
| Marine sites | | | | |
| All Scotland | 6.2 | ±3.1 | 0.012 | 0.82 |
| South Mainland | -3.4 | ±6.2 | 0.306 | 0.26 |
| North Mainland | 8.7 | ±5.4 | 0.026 | 0.75 |
| Shetland | 7.4 | ±3.7 | 0.014 | 0.82 |
| Orkney | 11.5 | ±3.7 | 0.003 | 0.91 |
| Outer Hebrides | 10.6 | ±7.1 | 0.013 | 0.71 |
| Freshwater Sites | | | | |
| All Scotland | 2.0 | ±1.5 | 0.051 | 0.66 |
| South Mainland | 1.9 | ±1.3 | 0.039 | 0.69 |
| North Mainland | 0.4 | ±1.9 | 0.663 | 0.05 |
| Shetland | 6.5 | ±3.9 | 0.026 | 0.75 |
| Orkney | 2.4 | ±2.3 | 0.097 | 0.54 |
| Outer Hebrides | 3.0 | ±2.2 | 0.041 | 0.69 |

[a]IPNV, infectious pancreatic necrosis virus; CI, confidence intervals; p, probability; $r^2$, coefficient of determination for bivariate analysis

from very low levels to high levels, even to near ubiquity in Shetland salmon farms. IPNV prevalence has typically increased annually by 10% in saltwater and by 2% to 3% in freshwater. In freshwater in Shetland, the annual increase was 6.5%.

In spite of IPNV's emergence in most of Scotland, the virus's prevalence did not change significantly in southern areas; if anything, it declined. The reasons for this are unclear, but the pattern suggests that controlling the emergence of IPNV may be possible. In Sweden, IPNV is controlled by culling, and the virus is officially absent from that country in spite of very high levels in neighboring Norway (11). Notably, in 1999 or 2000 in many areas of Scotland, marine IPNV prevalence declined, or at least increased less rapidly. This decline followed a period of fallowing, which in turn followed widespread culling to control the outbreak of infectious salmon anemia of 1998–99 (22,4).

Multilevel modeling shows that the regional pattern dominates the structure of variation in IPNV's prevalence in salmon farms, overshadowing even the difference between marine and freshwater sites, although this could be slightly underestimated. The strength of this regional pattern is due to the extremely high prevalence of IPNV in Shetland and its low prevalence in the Outer Hebrides in both freshwater and saltwater sites.

The regional variation is in decline for marine salmon farms, however. As IPNV approaches ubiquity in Shetland, the virus has reached the saturation point; in many other areas, the increase remains rapid. In the Outer Hebrides, where prevalence was lowest, the increase has been the most rapid. As a result, IPNV prevalence levels are converging, and the standard

deviation among regional means has declined. As the regions have become increasingly similar, regional differentiation in control policy becomes less useful.

Our analysis is not intended to describe the absolute prevalence of IPNV in Scotland. Detection limits (9,10) and, on occasion, small numbers of fish (19) may mean that infection may not always be detected at an infected site; thus, the true prevalence of IPNV within samples is probably higher than reported. Uncertainty in test results is also a fundamental limitation (10) and would apply to the results of a survey specifically designed to determine viral prevalence as much as it applies to these monitoring data. Sample site selection is not random but is based on the need to monitor for IPNV, which may also lead to biases. However, with a centralized organization that collects data by standardized methods, trends and regional variation may be analyzed in relative terms.

Improved analysis methods may have contributed to increases in reported IPNV prevalence in marine water in 2001;



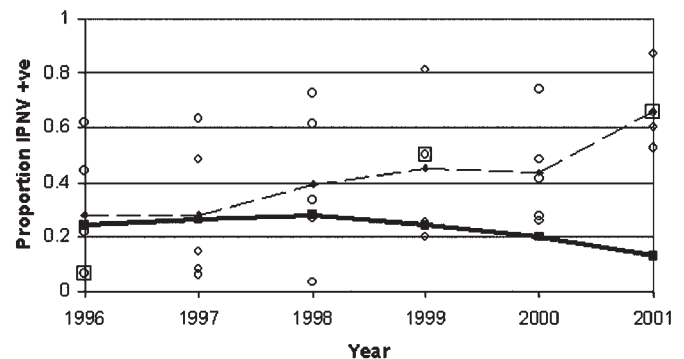Figure 4. Mean of regional annual mean prevalence of infectious pancreatic necrosis virus in marine salmon farms (thin dashed line) and standard deviation of regional annual means (thick solid line), by year. Individual marine regional annual means are shown by circles (where two circles overlay, a large square is added). Standard deviation has fallen at an increasing rate, in spite of increasing mean regional prevalence.

Table 3. Variance in the error terms obtained in a multilevel model with binomial assumptions regarding infectious pancreatic necrosis virus distribution

| Level of model | Variance standard error | Component |
|---|---|---|
| 1 | 0.333 ± 0.474 | Saltwater/freshwater |
| 2 | 0.582 ± 0.341 | Region |
| 3 | 0.362 ± 0.111 | Year |
| 4 | 0.042 ± 0.055 | Season |

however, this increase continues a trend and reflects an increase in clinical IPN cases (11,15). Increased experience per se is unlikely to be a factor in increased detection during the study period since the FRS Marine Laboratory has been sampling for IPNV since the 1970s (18) and has contributed to the development of standard tests (9). Moreover, prevalence shows different temporal patterns in different areas, in spite of centrally controlled methods and personnel. These increases therefore cannot be explained in terms of improvements in methods, nor can differences in methodology be used to explain regional differences in prevalence. In addition, sampling methods used in freshwater may be slightly more sensitive than those used for marine sites, which could mean the difference in prevalence between fresh and marine waters is even larger than reported here. Because IPNV has a wide variety of host species (7,13), persistent carriers among recovered hosts (23), and the ability to be carried by piscivorous bird vectors (24), the virus is difficult to eradicate, once established. Given IPNV prevalence increases of approximately 10% per year, effective control would have to be reestablished very soon if IPNV is not to become as ubiquitous in most areas as it is in Shetland. Owing to limitations in detection methods, IPNV prevalence in samples may be even higher than reported (10). IPNV prevalence has been held steady in southern areas of Scotland, indicating that an increase is not inevitable; Sweden has maintained IPNV-free status. In freshwater sites in Scotland, IPNV is less prevalent, and the prevalence is increasing at lower rates; therefore, control can likely be reasserted in freshwater.

IPNV is one of a series of emerging pathogens causing serious economic damage to aquaculture in Scotland (6,22) and around the world (1,2). Although data collected from programs to monitor these pathogens are not always statistically unbiased, they form a valuable resource on emerging and established (8) diseases. As further fish species are cultivated (e.g., cod *Gadus morhua* [3,7]), such data are required to design a sustainable industry.

## Acknowledgments

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

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Address for correspondence: A.G. Murray, FRS Marine Laboratory, Victoria Road, Aberdeen AB11 9DB, U.K.; fax: 01224 295511; email: murrays@marlab.ac.uk

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The Quarantine of Dubrovnik

# Molecular Epidemiology of Human Enterovirus 71 Strains and Recent Outbreaks in the Asia-Pacific Region: Comparative Analysis of the VP1 and VP4 Genes

Mary Jane Cardosa,* David Perera,* Betty A. Brown,† Doosung Cheon,‡ Hung Ming Chan,* Kwai Peng Chan,§ Haewol Cho,‡ and Peter McMinn¶

This study provides a comprehensive overview of the molecular epidemiology of human enterovirus 71 (HEV71) in the Asia-Pacific region from 1997 through 2002. Phylogenetic analysis of the VP4 and VP1 genes of recent HEV71 strains indicates that several genogroups of the virus have been circulating in the Asia-Pacific region since 1997. The first of these recent outbreaks, described in Sarawak (Malaysian Borneo) in 1997, was caused by genogroup B3. This outbreak was followed by large outbreaks in Taiwan in 1998, caused by genogroup C2, and in Perth (Western Australia) in 1999, where viruses belonging to genogroups B3 and C2 cocirculated. Singapore, Taiwan, and Sarawak had HEV71 epidemics in 2000, caused predominantly by viruses belonging to genogroup B4; however, large numbers of fatalities were observed only in Taiwan. HEV71 was identified during an epidemic of hand, foot and mouth disease in Korea; that epidemic was found to be due to viruses constituting a new genogroup, C3.

Human enterovirus 71 (HEV71) was first isolated in 1969 (1) and is most often associated with outbreaks of the mild childhood exanthem, hand, foot and mouth disease (HFMD) (2). HEV71 is closely related to coxsackie virus A16 (CA16), the other major causative agent of HFMD, but unlike CA16, HEV71 is also associated with cases of acute neurologic disease including poliomyelitis-like paralysis, encephalitis, and aseptic meningitis (3,4). Although HEV71 outbreaks associated with small numbers of cases of severe neurologic disease were reported in the 1980s in Australia (5), Asia (6), and the United States (4), mortality associated with such outbreaks was low, unlike earlier outbreaks in Bulgaria in 1975 (7) and Hungary in 1978 (8). Twenty years later in 1997, deaths associated with epidemics of HEV71-associated HFMD in Sarawak, Malaysia (9), followed closely by outbreaks with

high mortality in Taiwan in 1998 and 2000 (10,11), have raised considerable public concern about the virulence of this virus and the disease syndromes most recently attributed to it (12–14).

Several groups have attempted to describe the molecular epidemiology of HEV71 in the Asia-Pacific region, and the phylogenetic relationships of recent HEV71 strains to earlier strains from Asia and other parts of the world have been described (15–19). In the past 4 years, nine publications have presented comparative analyses of HEV71 strains based on several genome regions (Table 1). Taken together, more than 250 HEV71 strains isolated from the region between 1997 and 2002 have been analyzed by different groups. However, gaining a comprehensive insight into the phylogenetic relationships between all these HEV71 strains has been difficult because of the lack of a standardized methodology. The strategies used by each of these groups are summarized in Table 1.

We sequenced the complete VP1 gene of 66 HEV71 isolates from Singapore, Sarawak (Malaysian Borneo), and Perth, Western Australia, from 1997 to early 2001 (22) and determined that these recent strains were from the genogroups B and C, based on the nomenclature of Brown et al. (21). In Sarawak in 1997, isolates from fatal and nonfatal cases all belonged to genogroup B, in a previously undescribed cluster that we have named B3 (22). Viruses belonging to genogroup B3 were also isolated in Singapore in 1998, and B3 was the genogroup most commonly identified for viruses isolated in Perth during 1999. However, HEV71 strains isolated from children with severe neurologic disease during the Perth epidemic belonged to genogroup C2 (22). Another previously undescribed genogroup B cluster (B4) was identified in Singapore in 1997 and continued to circulate there in 2000 (23) through 2002. Viruses from genogroup B4 were also identified as the primary cause of a large HFMD outbreak in Sarawak in 2000.

As several regions of the HEV71 genome have been used in phylogenetic studies, including the VP1 (21,22) and VP4 (15,19) genes and the 5′ untranslated region (5′ UTR) (16,20),

*Universiti Malaysia Sarawak, Sarawak, Malaysia; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡National Institute of Health, Seoul, Korea; §Singapore General Hospital, Singapore; and ¶Telethon Institute for Child Health Research, Perth, Western Australia, Australia

Table 1. Summary of recent studies of human enterovirus 71 (HEV71) phylogeny

| Reference | Gene/ region[a] | No. of nucleotides sequenced | No. of new strains (source, time) |
|---|---|---|---|
| AbuBakar et al., 1999 (20) | 5′ UTR | 440 | 13 (Malaysia, 1997) |
| Brown et al., 1999 (21) | VP1 | 891 | 113 (several countries, 1970–1998) |
| Shimizu et al., 1999 (15) | VP4/VP2 | 420 | 29 (Malaysia and Japan, 1997; Taiwan, 1998; 5 others (Bulgaria, Hungary, Japan, Taiwan, USA), 1973–1980) |
| Wang et al., 2000 (16) | 5′ UTR | 681 | 36 (Taiwan, 1998) |
| Shih et al., 2000 (17) | VP1 | >500 | 16 (Taiwan, 1998) |
| Singh et al., 2000 (18) | VP1 | 341 | 20 (Singapore, Japan, and Peninsular Malaysia, 1997–1998) |
| Chu et al., 2001 (19) | VP4 | 207 | 20 (Taiwan, 1998); 3 (Taiwan, 1986) |
| McMinn et al., 2001 (22) | VP1 | 891 | 66 (Sarawak, Singapore, and Perth, 1997–2001) |
| Wang et al., 2002 (11) | 5′ UTR VP1 | 648 841 | 26, 3, and 19 strains (Taiwan, 1998, 1999, and 2000) were sequenced in the 5′ UTR; 12, 2, and 19 strains (same 3 years) were sequenced in VP1 |

[a]UTR, untranslated region.

undertaking a comprehensive analysis of the molecular epidemiology of recent HEV71 activity in the Asia-Pacific region has not been possible. To overcome this deficiency, we present here a comprehensive phylogenetic analysis of recent HEV71 strains, accomplished by examining both the VP1 and VP4 genes.

## Materials and Methods

### Viruses

All viruses used in this study were propagated in rhabdomyosarcoma cells before extraction of RNA. Thirty-nine representative HEV71 isolates from recent years in Sarawak, Singapore, Perth, and Korea, as well as 16 strains isolated in the United States from 1972 to 1995, were propagated in rhabdomyosarcoma cells and subjected to nucleotide sequence analysis (see below). These strains were used to generate 12 new VP1 gene sequences and 55 new VP4 gene sequences (Appendix Table 1, online only).

### Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

RNA was extracted from infected rhabdomyosarcoma cell supernatants by using the High Pure viral nucleic acid kit (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was performed as described (22), except that the annealing temperature for amplification of the VP4 gene was 50°C. Primer pairs 159/162 and 161/NP1A (24) were used for VP1 amplification of nucleotides (nt) 2385 to 2850 relative to BrCr; for VP4 amplification, the primers VP2-REV (5′ TTCCAAT-ACCACCCCTTGGATGA 3′) and EVP-2 (19) were used to amplify nt 449 to 1192 relative to BrCr.

### Nucleotide Sequence Analysis

PCR products were purified from gels with the QIAquick gel extraction kit (QIAGEN Inc, Valencia, CA). Cycle sequencing was achieved with the primers 159/162 and 161/NP1A for VP1 and EVP-4 (15) and VP2-REV for VP4 by using the Big Dye Terminator Cycle Sequencing kit version 2.0 (Applied Biosystems, Foster City, CA). All sequences were determined for both strands by using the ABI377 automated DNA sequencer (Applied Biosystems).

### HEV71 Sequence Data Obtained from GenBank

The VP4 gene sequences of another 78 HEV71 strains obtained from GenBank (Appendix Table 2, online only) were included in this analysis, allowing the generation of dendrograms containing 128 HEV71 strains isolated from 1970 to 2002. These strains were isolated in the United States, Japan, Taiwan, Malaysia, Singapore, China, Bulgaria, Hungary, and the United Kingdom. In addition, 33 complete and 9 near-complete VP1 gene sequences of HEV71 strains retrieved from GenBank and used in this study are listed in Appendix Table 2, online only.

### Phylogenetic Analysis

Alignment of the VP1 and VP4 gene sequences was undertaken by using the ClustalW program (25). Dendrograms were constructed by using the neighbor-joining method with PHYLIP, version 3.5 (26) and drawn using TreeView (27). Bootstrap analysis with 1,000 pseudoreplicates was performed by using the program Seqboot (28). The CA16 strain G10 (29) was used as an outgroup for phylogenetic analysis of the VP4 sequence data, and the HEV71 prototype strain BrCr-CA-70 (30) was used as an outgroup for analysis of the VP1 sequence data. Historically Brown and co-workers (21) described genogroups as lineages of HEV71 distinguished by differing at least 15% in the VP1 gene. In this study we maintained the genogroups originally described and designated subgenogroups to aid in discussing evolving progeny viruses.

## Results

### Overview of HEV71 Phylogeny

Figure 1 presents an overview of the VP4-based phylogenetic tree, generated by including representative members of each of the genogroups A, B, and C. While there are a few outliers, this comprehensive VP4-based dendrogram accurately reproduces the genogroup clusters B1, B2, B3, B4, C1, and C2 (21,22) supported by the bootstrap values indicated. However,
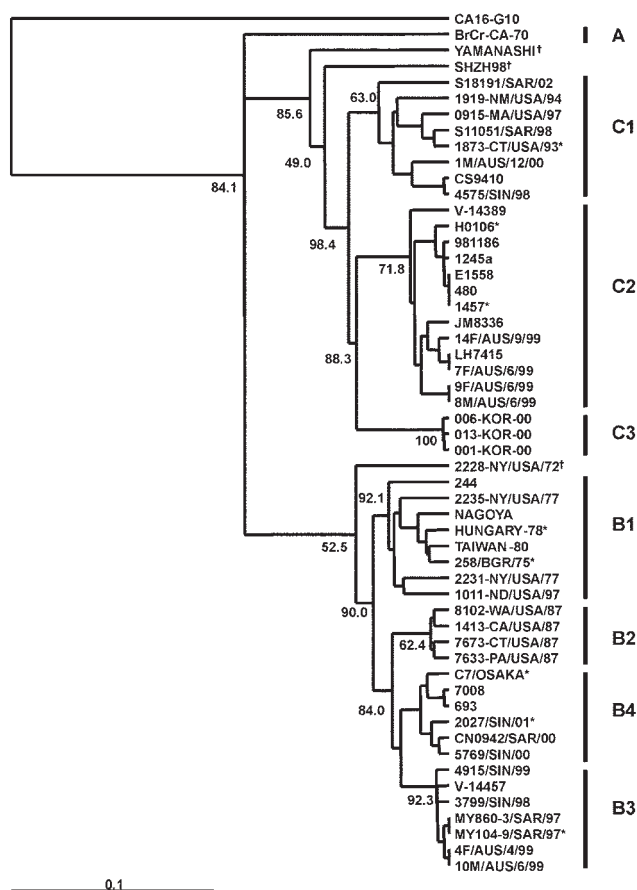
Figure 1. An overview of the genetic relationships of human enterovirus 71 (HEV71) strains isolated from 1970 through 2002. Dendrogram showing the genetic relationships among 53 HEV71 strains based on the alignment of the complete VP4 gene sequence (nucleotide positions 744–950). Details of the HEV71 strains included in the dendrogram are provided in Appendix Tables 1 and 2 (online only). Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The VP4 nucleotide sequence of coxsackie virus A16 (CA16) (29) was used as an outgroup in the analysis. *Denotes HEV71 isolates from fatal cases; †denotes HEV71 strains falling outside existing genogroup boundaries.

the Korean isolates form a distinct cluster in genogroup C with high bootstrap support, which we have designated genogroup C3. Selected HEV71 strains from the collection of the Centers for Disease Control and Prevention (CDC) were used to anchor the tree to maintain consistency of nomenclature between our VP4-based tree and the VP1-based tree published earlier by Brown et al. (21). The viruses in genogroups B and C share at least 78.3% nucleotide sequence identity. Within the B genogroup, the strains share at least 87.9% identity; the strains within the C genogroup share at least 84.5% identity. Overall, the C genogroup has greater diversity than the B genogroup. Within all subgenogroups, virus strains have >90% nucleotide sequence identity (Table 2). The divergence between genogroups A and B as well as A and C is 20% to 21%; the divergence between genogroups B and C is 16% to 25%. The

strains in our collection from two of the more recently described genogroups B3 and C3 showed very high similarity to each other within the genogroup (>98% and 99% identity, respectively), while those within the B1 and C1 genogroups showed the widest divergence. The phylogenetic relationships between the consensus sequences of the different genogroups based on VP4 analysis are shown as an unrooted tree (Figure 2). This cladogram clearly illustrates that the C genogroup viruses are more divergent than the B genogroup viruses; however, because we might not have strains that are evenly distributed temporally and geographically, we were unable to determine if this difference in divergence means that genogroup B viruses have evolved more recently than the genogroup C viruses.

## Genogroup B

The four different genogroup B clusters described in our earlier VP1-based study (23) were accurately reproduced with VP4 gene sequences (Figure 3). This approach allows the inclusion of HEV71 strains from Taiwan and Japan published by Shimizu et al. (15) and Chu et al. (19). We show that, in the 1998 Taiwan epidemic, HEV71 strains belonging to genogroup B4 cocirculated in small numbers (e.g., 7008/98, 5929/98, and J1263P4). These strains are genetically similar to B genogroup viruses circulating in Japan during 1997 (V-14433, V-14429, C7/Osaka) but are distinct from the B3 genogroup of viruses circulating at the same time in Sarawak (e.g., MY104-9/SAR/97 and SK-EV006). Genogroup B4 strains have been circulating in the Asia-Pacific region at least since 1997, as these strains were isolated sporadically in Japan and Singapore in 1997 and in Taiwan during 1998, before the large outbreaks in 2000 in Singapore, Sarawak, and Taiwan associated with viruses from this genogroup. HEV71 strains of the B4 genogroup continued to be isolated in Singapore during 2001 and 2002.

A Taiwanese group reported high death rates during an HEV71 outbreak in 2000 (11). Using phylogenetic analysis based on 840 nt of the VP1 gene (full length 891 nt), these investigators described a shift in genogroup from C in 1998 to B in 2000. To place the most recent Taiwanese strains in a regional context, we have included four representative genogroup B strains from the 2000 outbreak in Taiwan as well as two Taiwanese genogroup B strains from 1998 and 1999 in a limited VP1 gene-based dendrogram, together with representative strains from genogroups B1, B2, B3, and B4 (Figure 4).

Table 2. VP4 nucleotide sequence identities within genogroups

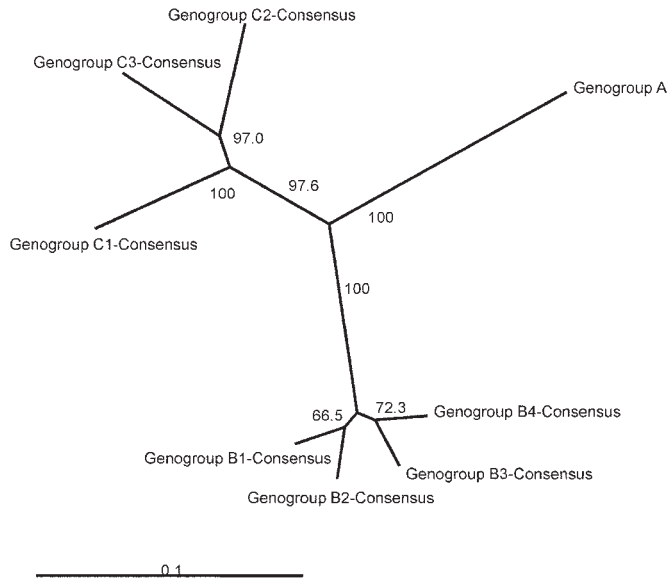| Genogroup | % Identity |
|---|---|
| B1 | 91.3 to 100 |
| B2 | 96.6 to 98.6 |
| B3 | 98.1 to 100 |
| B4 | 94.7 to 100 |
| C1 | 90.3 to 100 |
| C2 | 93.2 to 100 |
| C3 | 99.0 to 100 |

Figure 2. An overview of the genetic relationships of human enterovirus 71 (HEV71) strains isolated from 1970 through 2002. Unrooted clado-gram shows the genogroup relationships of HEV71 based on an alignment of the complete VP4 gene (nucleotide positions 744–950) consensus sequences for genogroups B1, B2, B3, B4, C1, C2, and C3. The complete VP4 gene sequence of the prototype strain BrCr-CA-70 (30) was used as an outgroup in the analysis. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance.

This analysis clearly identifies the Taiwan 2000 strains as belonging to genogroup B4. The temporal shift of B4 genogroup strains that occurred from one lineage in 1997 through 1999 (in Taiwan and Singapore) to a second lineage in 2000 through 2001 (in Taiwan, Singapore, and Sarawak) gave rise to large epidemics in these countries in 2000.

Another notable point that arises from VP4 gene-based phylogenetic analysis of genogroup B is that some older U.S. strains (2228-NY72 and 6910-OK87), which clearly belong within genogroup B1 in VP1-based analysis (21), do not lie within B1 in the VP4-based dendrogram (Figure 3). Moreover, in the United Kingdom a similar B genogroup "outlier" was isolated as recently as 1999. These outliers in the VP4-based analysis are very closely related to the B1 cluster, and this discrepancy probably results from the lower discriminating power obtained when using the VP4 gene for this analysis, as evidenced by lower bootstrap values at the major nodes.

## Genogroup C

Phylogenetic analysis that uses VP4 gene sequences also confirms previous observations that the major strains circulating in the Taiwan outbreak of 1998 were from genogroup C, more specifically C2, following the nomenclature of Brown et al. (21) (Figure 5). The Taiwanese isolates of 1998 clustered closely together in a lineage related to C2 strains isolated in Japan in the previous year and to those causing severe neurologic disease in Perth in 1999. This finding is supported by

VP1-based analysis of Taiwanese strains from 1998 (Figure 6). We also located in GenBank a number of VP4 gene sequences of strains isolated in the United Kingdom between 1997 and 1999. These strains formed a cluster within genogroup C2, together with strains from Japan and Australia during 1997 and 1999, respectively. In our previous VP1-based study, the Australian C2 genogroup strains isolated during 1999 formed two distinct lineages (22), which are reproduced in the VP4 analysis. Similarly, Japanese isolates from 1997 form distinct lineages within genogroup C2.

An outbreak of HFMD with cases of aseptic meningitis and paralysis occurred in Korea during 2000; 11 strains of HEV71 from this outbreak are included in this study. All Korean strains formed a distinct cluster in genogroup C and showed 90% to



Figure 3. Phylogenetic relationships of human enterovirus 71 (HEV71) strains belonging to genogroup B (21). Dendrogram shows the genetic relationships among 56 HEV71 strains belonging to genogroup B, based on the alignment of the complete VP4 gene sequence (nucleotide positions 744-950). Details of the HEV71 strains included in the dendrogram are provided in Appendix Tables 1 and 2 (online only). Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The VP4 nucleotide sequence of coxsackie virus A16 (CA16) (29) was used as an outgroup in the analysis. *Denotes HEV71 isolates from fatal cases; †Denotes HEV71 strains falling outside existing genogroup boundaries.

Figure 4. Phylogenetic relations of human enterovirus 71 (HEV71) strains belonging to genogroup B (21). Dendrogram shows the genetic relationships among 24 HEV71 strains belonging to genogroup B, based on the alignment of a partial VP1 (nucleotide positions 2442–3281) or complete VP1 (nucleotide positions 2442–3332) gene sequences. Details of the HEV71 strains included in the dendrogram are provided in Appendix Tables 1 and 2 (online only). Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The VP1 nucleotide sequence of the prototype BrCr-CA-70 (30) was used as an outgroup in the analysis.

The Japanese 1978 isolate "Yamanashi" is an outlier of the C genogroup. Twenty years later (1998), another C genogroup outlier was isolated in China (SHZH98). Both of these strains appear to be ancestral to C1, C2, and C3, raising the possibility that China may still have ancestral strains of HEV71 currently circulating.

## Discussion

Although several studies have attempted to describe the phylogenetic origins of HEV71 strains recently circulating in the Asia-Pacific region (11,15,17–22), no single study has included representative virus strains from all of the HEV71 epidemics recorded in the Asia-Pacific region since 1997 in a comprehensive analysis. We attempted to provide a more complete picture of the phylogenetic relationships between the
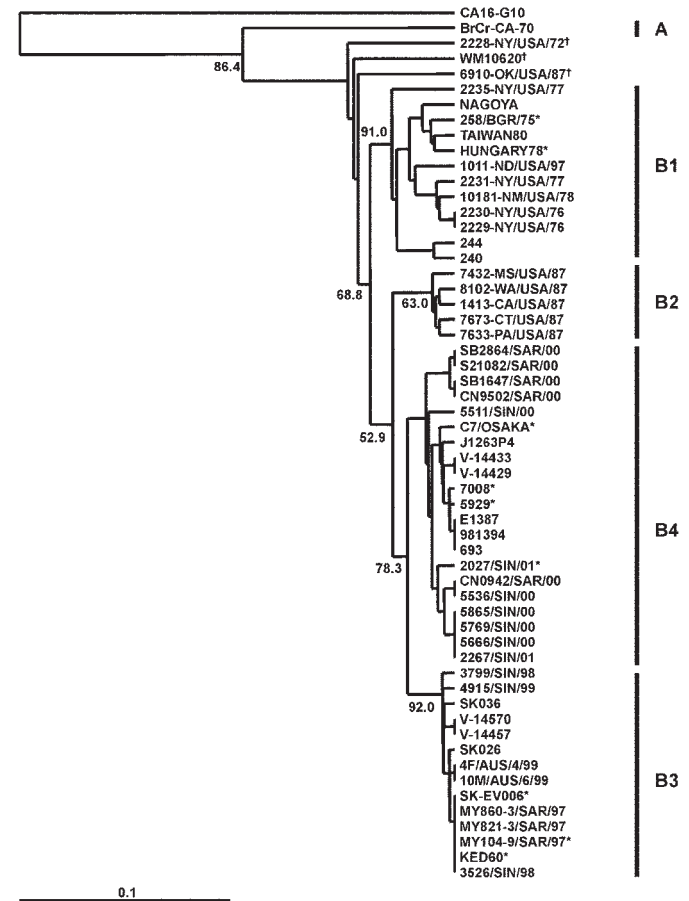


Figure 5. Phylogenetic relationships of human enterovirus 71 (HEV71) strains belonging to genogroup C (21). Dendrogram shows the genetic relationships among 74 HEV71 strains belonging to genogroup C, based on the alignment of the complete VP4 gene sequence (nucleotide positions 744–950). Details of the HEV71 strains included in the dendrogram are provided in Tables 2 and 3. Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The VP4 nucleotide sequence of coxsackie virus A16 (29) was used as an outgroup in the analysis. *Denotes HEV71 isolates from fatal cases; †Denotes HEV71 strains falling outside existing genogroup boundaries.
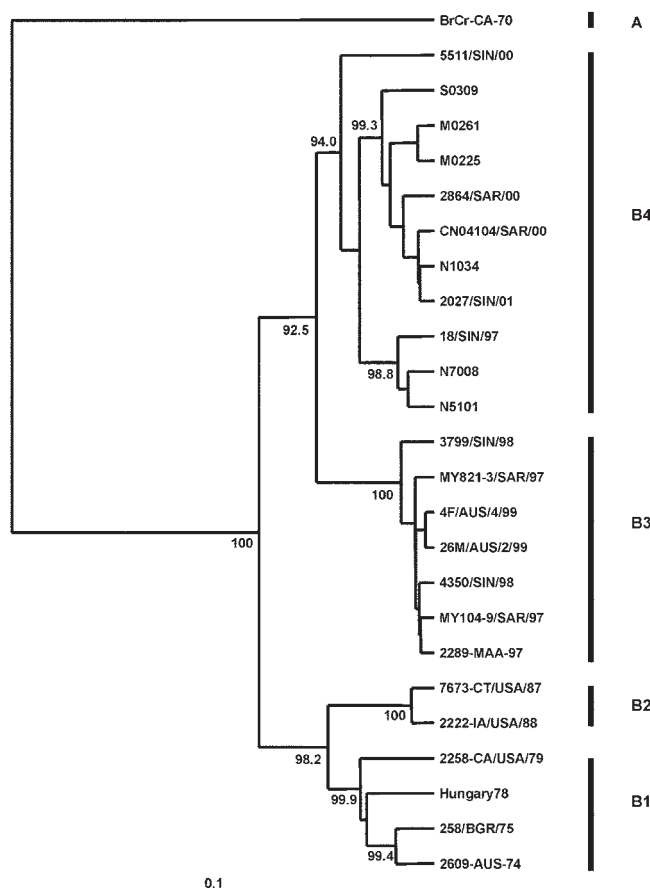
92% nt sequence identity with C1 and C2 subgroups and 79% to 82% identity with the different genogroup B strains. A dendrogram comparing the VP1 gene sequences of a limited number of HEV71 genogroup C strains with the 11 Korean isolates from 2000 and the single 2002 isolate from Sarawak is shown in Figure 6. This VP1-based dendrogram supports the VP4-based analysis, suggesting that the Korean HEV71 isolates form a distinct cluster, which we have designated C3. The Sarawak strain isolated in 2002 is from the C1 genogroup. Chu et al. (19) described the major genogroup circulating in Taiwan in 1998 as C3. However, because of the extent of variation in clusters, our previous analysis based on the VP1 gene designates the Taiwan outbreak strain as belonging to genogroup C2 (22); this current study establishes the designation C3 for the new Korean strains isolated during 2000.
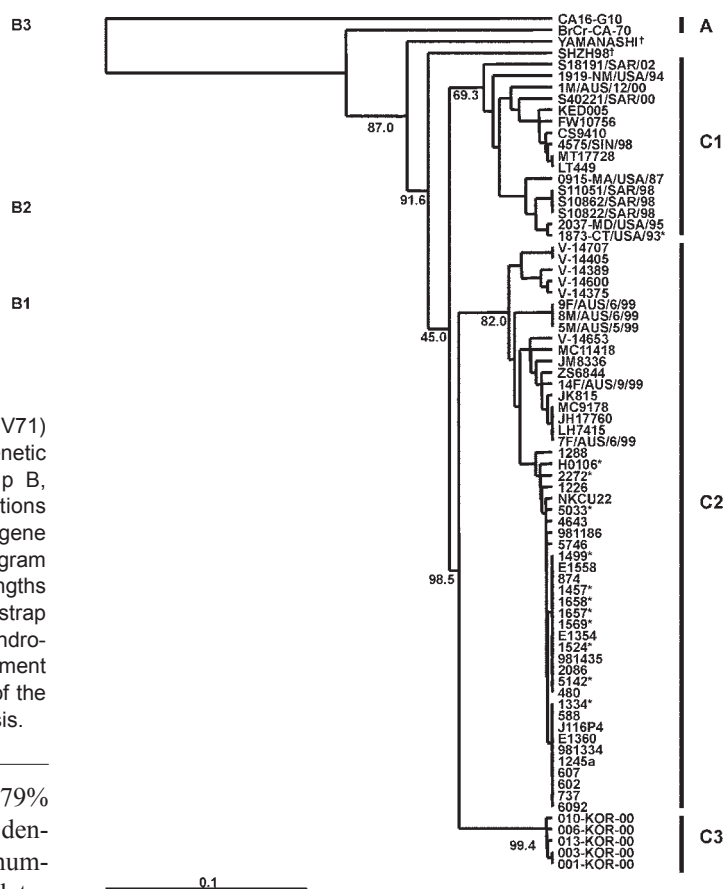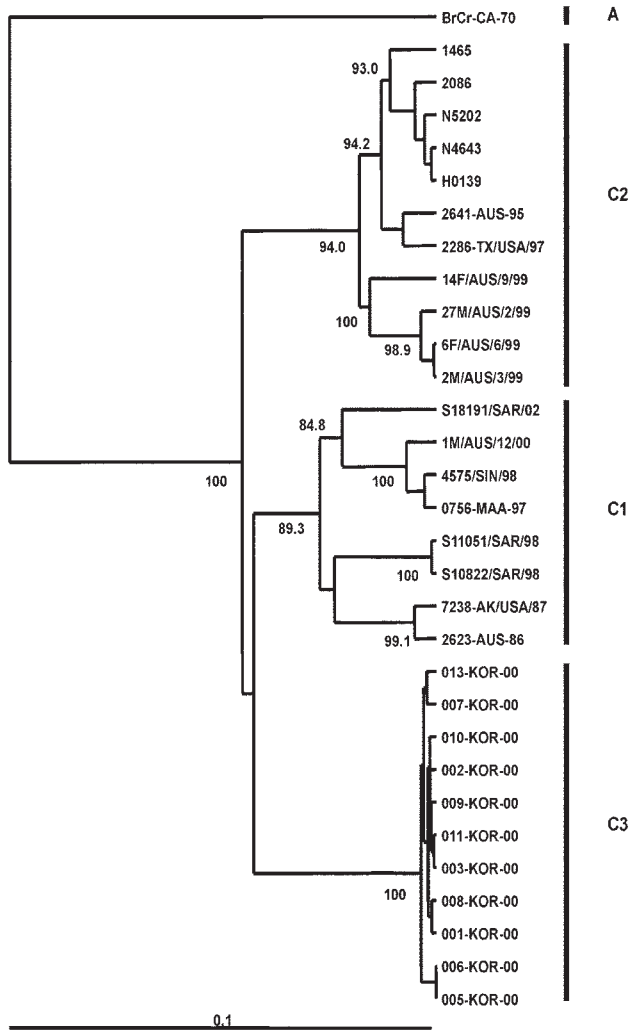
Figure 6. Phylogenetic relationships of human enterovirus 71 (HEV71) strains belonging to genogroup C (21). Dendrogram shows the genetic relationships among 30 HEV71 strains belonging to genogroup C, based on the alignment of a partial VP1 (nucleotide positions 2442–3281) or complete VP1 (nucleotide positions 2442–3332) gene sequences. Details of the HEV71 strains included in the dendrogram are provided in Tables 2 and 3. Branch lengths are proportional to the number of nucleotide differences. The bootstrap values in 1,000 pseudoreplicates for major lineages within the dendrogram are shown as percentages. The marker denotes a measurement of relative phylogenetic distance. The VP1 nucleotide sequence of the prototype BrCr-CA-70 (30) was used as an outgroup in the analysis.

HEV71 strains circulating in recent outbreaks and to place them in the context of strains isolated earlier and in other parts of the world since this virus was first described. The different genetic typing strategies and nomenclatures adopted by different groups have led to much confusion about the molecular epidemiology of recent HEV71 strains. Briefly summarizing these studies and linking the data presented into a single unifying picture are thus necessary.

Two very limited phylogenetic studies focused on short segments of the 5′ UTR. The first compared 13 strains of HEV71 isolated from fatal and nonfatal cases in Malaysia during 1997

(20) and showed that at least two lineages of this enterovirus circulated in Peninsular Malaysia during that period. HEV71 strains isolated from a pair of siblings, one with a fatal illness and one with uncomplicated HFMD, belonged to different genetic lineages. Wang et al. (16) also examined the 5′ UTR of 36 isolates from the large Taiwanese epidemic in 1998 and reported that most of the isolates clustered into a single genetic lineage, with two exceptions. Both of these studies are of limited value, not only because of the narrow focus on isolates from a single country in a single year, but also because the highly conserved 5′ UTR is not a suitable region upon which to base phylogenetic analysis of enteroviruses (31).

More recently, Wang et al. (11) examined the 5′ UTR and the VP1 gene of 58 Taiwan strains isolated in 1998–2000 and showed that the major genogroup of HEV71 circulating in Taiwan changed from genogroup C in 1998 to genogroup B in 1999–2000. In this study, we reexamined nine HEV71 strains reported by Wang et al. and found that the major genogroup circulating during the 1998 Taiwan epidemic was C2, as we had shown previously (22). More importantly, our analysis shows that the major genogroup circulating in Taiwan changed from C2 in 1998 to B4 in 2000.

Two other phylogenetic analyses of HEV71 focus on the VP4 gene region. Shimizu et al. (15) examined a 420-nt region in the VP4/VP2 gene region of 16 isolates from Malaysia and Japan in 1997 and 13 isolates from Taiwan in 1998. These recent isolates were distributed into two major genogroups, which these researchers labeled genotypes A and B. (These genotype designations are not equivalent to genogroups A and B, originally described by Brown et al. [21]). Chu et al. (19) sequenced a 207-nt region of the VP4 gene of 23 Taiwanese strains. Twenty of the strains used in this study were isolated from fatal and nonfatal cases during the 1998 epidemic. Three strains isolated during the 1986 outbreak of HEV71-associated HFMD in Taiwan were included; this study confirmed that most 1998 Taiwanese strains were from a single genogroup, although several isolates were genetically distinct from the main group. We reexamined the VP4 genetic sequences from the HEV71 strains isolated in Taiwan during 1986 and found that they belonged to genogroup B1.

Use of the VP1 gene has been reported in four phylogenetic studies of HEV71 strains derived from recent HFMD epidemics in the Asia-Pacific region. Two of these studies appear to be of limited value, having analyzed only short VP1 gene sequences and limited numbers of HEV71 isolates within restricted geographic areas. Singh et al. (18) presented a dendrogram based on a 341-nt region of the VP1 gene and concluded that several recent Southeast Asian strains belonged to two completely new genotypes. However, this finding appears to be flawed, as closer examination of the sequences used in construction of the dendrogram shows that they include a mixture of partial VP1 gene sequences and 5′ UTR sequences from (occasionally) the same strain. Shih et al. (17) examined partial VP1 gene sequences from 16 isolates from fatal and nonfatal cases in Taiwan during 1998; Shih's study provided further

confirmation that most isolates from the 1998 Taiwan outbreak belonged to a single genogroup. Shih et al. (17) described this predominant genogroup as "genotype B" and the minor genogroup as "genotype C" but labeled the isolates in their dendrograms in the reverse order. If one assumes that the dendrogram labeling is correct, their findings are consistent with the nomenclature of Brown et al. (21).

In our study, we used all available published data in addition to new sequence data from the VP4 gene of 51 recent strains and the VP1 gene of 11 Korean strains and 1 Sarawak strain of HEV71 to provide a comprehensive picture of the molecular epidemiology of HEV71 in the Asia-Pacific region since 1997. Our study shows that the first recent HEV71 outbreak recognized in the region occurred in Sarawak in 1997 and was associated with the previously undescribed genogroup B3. Viruses belonging to this genogroup were also circulating in Singapore and Japan in 1997, continued to circulate in Singapore in 1998, and were the primary cause of the epidemic in Western Australia in 1999. Since 1999, genogroup B3 viruses have not been identified anywhere within the region. In 1998, viruses of the C2 genogroup were the primary cause of the Taiwanese epidemic; strains from a distinct lineage within the same genogroup also circulated in Western Australia during 1999. Viruses belonging to the C1 genogroup appear to have undergone low-level endemic circulation in Malaysia, Singapore, and Perth between 1997 and 2002 and have not been associated with large-scale epidemics to date.

Our analysis shows that a great diversity of HEV71 strains circulate in the region and elsewhere and that no particular genogroup is specifically associated with severe disease. Since we were hampered by the fact that many studies failed to provide an accurate diagnosis for the cause of death in fatal cases of HEV71 infection, we suggest that identification of cases of encephalitis, poliomyelitis-like paralysis, or both is likely to provide a more accurate endpoint in the determination of disease severity associated with particular HEV71 genogroups. At this time, insufficient published data are available relating to cause of death to perform this analysis. Currently, the best available data on disease severity are from our previous study (22), in which viruses isolated from children with severe neurologic disease belonged exclusively to genogroup C2 and possessed a unique amino acid substitution in VP1 at position 170(A→V).

Genogroup B4 strains were isolated occasionally in Peninsular Malaysia, Singapore, and Taiwan from 1997 to 1999. In 2000, viruses from this genogroup caused large epidemics in Sarawak, Singapore, and Taiwan; however, in Korea, an outbreak of HFMD was caused by the new C3 genogroup. Table 3 shows the major HEV71 genotypes circulating in countries within the region since 1997. Clearly, HEV71 is a major emerging virus in the region. Because of this virus' potential for causing severe neurologic disease, we need to understand the factors that have led to the spread of this virus and the genetic factors that contribute to its neurovirulence and epidemic potential.

In conclusion, VP1 and VP4 gene sequences both provide similar phylogenetic information, but the higher bootstrap values seen in the VP1 dendrograms provide greater confidence, particularly when elucidating new genotypes. Thus, the use of the shorter VP4 gene may be helpful for HEV71 surveillance, but the VP1 gene should still be used for molecular epidemiologic research and for confirming data obtained with VP4-based analysis. Virus identification and classification have been reliant on antigenic methods for serotypic identification, and VP1 gene sequence data have been shown to infer serotype (32). Furthermore, the protein encoded by the VP1 gene is the most exposed and immunodominant of the capsid proteins (33) and is likely to give the most useful information in molecular epidemiologic investigations, unlike untranslated sequences or genes, which encode products not found exposed on the virion surface.

**Author's Note**

Sarawak is currently experiencing a large outbreak of HEV71 that began in February 2003. Most cases have uncomplicated HFMD, but a small number include neurologic disease. With methods described in this article, more than 70 strains isolated this year have been sequenced in the VP4 gene region. The results show that 80% of the strains belong to genogroup C1(which has been seen sporadically in the Asia-Pacific region since 1997) and suggest that the next big outbreak of HEV71 in the region will likely be due to strains from this genogroup. The remaining strains belong to a distinct cluster within the B genogroup and may represent an emerging new subgenogroup.

Table 3. Summary of human enterovirus 71 (HEV71) genotypes circulating in the Asia-Pacific region since 1997[a]

| | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 |
|---|---|---|---|---|---|---|
| Singapore | B3, B4 | B3, C1 | B3 | **B4** | B4 | C1, B4 |
| Sarawak, Malaysia | **B3** | C1 | No HEV71 | **B4**, C1 | No HEV71 | C1 |
| Perth, Australia | - | - | **B3, C2** | C1 | No HEV71 | No HEV71 |
| Japan | B3, B4, C2 | - | - | - | - | - |
| Taiwan | - | **C2**, B4 | B4 | **B4** | - | - |
| Korea | - | - | - | **C3** | No HEV71 | No HEV71 |

[a]Boldface type indicates genogroups causing large outbreaks; –, no data available; No HEV71, no HEV71 identified despite active surveillance.

Dr. Cardosa is a virologist working closely with public health professionals and clinicians to investigate viral diseases in Malaysian Borneo. Her specific interests are viral encephalitis and dengue.

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Address for correspondence: Mary Jane Cardosa, Institute of Health and Community Medicine, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia; fax: +60 82 671785; email: janecardosa@yahoo.co.uk

# Invasive Group B Streptococcal Infections in Finland: A Population-based Study

**Outi Lyytikäinen,\* J. Pekka Nuorti,\* Erja Halmesmäki,† Petteri Carlson,† Jukka Uotila,‡ Risto Vuento,‡ Tapio Ranta,§ Hannu Sarkkinen,§ Martti Ämmälä,¶ Anja Kostiala,¶ and Anna-Liisa Järvenpää†**

We analyzed surveillance data on group B streptococcus (GBS) infection in Finland from 1995 to 2000 and reviewed neonatal cases of early-onset GBS infection in selected hospitals in 1999 to 2000. From 1995 to 2000, 853 cases were reported (annual incidence 2.2–3.0/100,000 population). We found 32–38 neonatal cases of early-onset GBS disease per year (annual incidence 0.6–0.7/1,000 live births). In five hospitals, 35% of 26 neonatal cases of early-onset GBS infection had at least one risk factor: prolonged rupture of membranes, preterm delivery, or intrapartum fever. Five of eight mothers screened for GBS were colonized. In one case, disease developed despite intrapartum chemoprophylaxis. Although the incidence of early-onset GBS disease in Finland is relatively low, some geographic variation exists, and current prevention practices are suboptimal. Establishing national guidelines to prevent perinatal GBS is likely to reduce the incidence of the disease.

Group B streptococcus (GBS), a leading cause of invasive bacterial infections in newborns, also affects pregnant women and elderly persons (1–4). In the United States, several studies have reported the incidence of GBS infection in different demographic groups, and guidelines were developed and implemented for the prevention of neonatal infection in the 1990s (1–6). In European countries, however, few population-based data on GBS infection are available and no national guidelines have been published (7–10).

In the United States, the recommended strategies to prevent perinatal GBS disease include either a risk-based or screening-based approach (5). In the risk-based approach, women in labor who have risk factors for GBS transmission (e.g., fever, prolonged rupture of the membranes, or preterm delivery) are offered intrapartum chemoprophylaxis. In the screening-based approach, vaginal and rectal combined swabs are cultured from all pregnant women and tested for GBS carriage during 35 to 37 weeks' gestation. Those identified as GBS carriers are offered intrapartum chemoprophylaxis.

In Finland, laboratory-based surveillance for invasive bacterial infections, including GBS, began in 1995. To identify opportunities for prevention, we analyzed national GBS surveillance data from 1995 to 2000. To assess the proportion of cases that might have been prevented by using the risk-based or screening approaches, we reviewed birth histories of infants with early-onset GBS disease in five hospitals participating in a nosocomial infection surveillance network from 1999 to 2000. We also conducted two national surveys: one evaluating the microbiologic methods used to screen for GBS cultures in Finnish clinical microbiology laboratories and the other on current practices related to GBS screening and antibiotic use in Finnish hospitals with obstetric services.

## Methods

### Surveillance

Finnish clinical microbiology laboratories routinely notify the National Infectious Disease Registry of bacterial isolations from blood and cerebrospinal fluid. Each report includes the following information: isolation date, birth date, sex, specimen type, and treatment location. Multiple reports of the same case are combined in the database if they are received within 3 months of first isolation. A case is defined as isolation of GBS from blood or cerebrospinal fluid; early-onset neonatal disease is defined as that occurring in infants <7 days old and late-onset disease as that occurring in infants 7–89 days old.

### Additional Data Collection and Chart Review

Neonatal GBS cases that occurred in five hospitals from 1999 to 2000 were identified through hospital wide surveillance of nosocomial bloodstream infections in connection with the Finnish Hospital Infection Program (SIRO). We obtained data on deliveries, local guidelines for perinatal GBS prevention, and microbiologic data (e.g., screening method, number of specimens examined, and number of GBS-positive specimens). In cases of early-onset disease, the following data were abstracted from the medical records: prenatal GBS screening, intrapartum fever >38°C, prolonged rupture of membranes ≥18 h before delivery, preterm delivery at <37 weeks of gestation, receipt of intrapartum antibiotics, and outcome of illness.

### Calculation of Incidence Rates and Statistical Analysis

Data from the National Population Registry, including live births, from 1995 to 2000 were used as denominators to calculate age- and sex-specific incidence rates and early-onset and

*National Public Health Institute, Helsinki, Finland; †Helsinki University Central Hospital, Helsinki, Finland; ‡Tampere University Hospital, Tampere, Finland; §Päijät-Häme Central Hospital, Lahti, Finland; and ¶Jorvi Hospital, Espoo, Finland

late-onset neonatal disease rates. The average annual incidences during the surveillance period were calculated by using the total number of cases, population, and live births from 1995 to 2000. To evaluate trends, rates of GBS disease in different age and sex groups were calculated for each 6-month period from January 1995 to December 2000. Data were analyzed by using Epi Info software, version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and SAS software, version 8.2 (SAS Institute, Inc., Cary, NC). A Poisson regression model was used to assess whether the observed changes in the rates were statistically significant.

### Surveys

In February 2002, we sent structured questionnaires by electronic mail to 20 of the 28 Finnish clinical microbiology laboratories and by regular mail to all Finnish hospitals with obstetric services (n=38). The laboratories were asked about their methods for screening cultures for GBS and the hospitals about their GBS prevention policies.

### Results

From 1995 to 2000, a total of 853 cases of invasive GBS disease were identified. Of bacterial isolates, 96% were obtained from blood and 4% from cerebrospinal fluid. The average annual incidence was 2.8 cases per 100,000 population (range by year, 2.2–3.0) and varied from 1.8 to 4.0 by health district. In women aged ≥65 years of age, the incidence increased significantly, from 1.1 per 100,000 in 1995 to 8.2 per 100,000 in 2000 (p<0.01 by Poisson regression). No trends were identified in other age or sex groups.

Infants of <1 year of age had the highest rate and accounted for 272 (32%) of 853 of all GBS infections (Table 1); 211 (78%) of 272 were early-onset disease and 203 (96%) of 211 were identified during the first 2 days of life. The average annual incidence of early-onset infections was 0.6 per 1,000 live births (range by year, 0.6–0.7; 32–38 cases/y; Table 2) and varied from 0.1 to 1.3 by health district. In 7 of 20 health districts in the country, the average annual incidence was >0.7 per 1,000 live births. Among 211 early-onset cases, 98% of isolates were obtained from blood and 2% from cerebrospinal fluid. The average annualized incidence of late-onset infections was 0.2 per 1,000 live births (range by year, 0.1–0.3; 6–16 cases/y; Table 2) and varied from 0.0 to 0.4 by health district. Among 56 cases of late-onset GBS disease, 59% of isolates were obtained from blood and 41% from cerebrospinal fluid.

From 1999 to 2000, a total of 38,687 women delivered babies in the five study hospitals, accounting for one third of all live births in Finland. Of the deliveries, 20% were cesarean sections and 7% preterm deliveries. None of the hospitals had a policy for universal maternal screening of GBS. Their protocol included screening risk groups only and prescribing intrapartum prophylaxis for GBS-positive women. Patients who had previously delivered infants with GBS disease and who had tested positive for GBS bacteriuria during pregnancy were also screened. Two hospitals prescribed ampicillin, and three hospitals prescribed penicillin. To identify GBS carriers, four hospitals cultured the samples and one used an antigen test. Only vaginal swabs were collected. A total of 9,220 screening specimens were obtained; 12% of them were positive for GBS. The proportion of positive specimens varied from 4% to 21% in the five hospitals.

In the study hospitals, 26 cases of early-onset disease (0.7/1,000 live births) and four cases of late-onset disease (0.1/1,000 live births) were identified. One premature neonate died. Delivery occurred at 25 weeks of gestation, and the screening result was negative. Of 26 women who had infants with early-onset disease, 1 received intrapartum antibiotics because of prolonged rupture of the membranes and a positive screening result. Of the 25 women who did not receive intrapartum antibiotics, 18 (72%) were not screened. Eight (32%) developed at least one risk factor (six had duration of ruptured membranes ≥18 hours, two had delivery at <37 weeks of gestation, and one had intrapartum fever). Of the 18 women not screened, 4 (22%) showed risk factors at the time of labor (3 had duration of ruptured membranes ≥18 hours, and 1 had delivery at <37 weeks of gestation).

All 26 isolates from case of early-onset infection and 4 from cases of late-onset infection were evaluated for antibiotic susceptibility. All isolates were susceptible to penicillin; two isolates (8%) were resistant/intermediate to erythromycin, and one isolate (4%) was resistant to clindamycin.

### Surveys

All 20 microbiology laboratories responded. Of the laboratories, 13 (65%) had a specific laboratory request for GBS culture; 9 laboratories requested cultures from vagina (69%) and 8 (62%) from cervix. None of the laboratories recommended rectal cultures. One laboratory used selective broth media to culture GBS.

Table 1. Incidence of invasive group B streptococcus infection by age and sex, Finland, 1995–2000

| Age group (y) | Men | | Women | | Total | |
|---|---|---|---|---|---|---|
| | No. of cases | Rate[a] | No. of cases | Rate[a] | No. of cases | Rate[a] |
| <1 | 143 | 79.3 | 129 | 74.7 | 272 | 77.1 |
| 1–14 | 1 | 0.04 | 5 | 0.2 | 6 | 0.1 |
| 15–64 | 128 | 1.2 | 193 | 1.9 | 321 | 1.6 |
| >64 | 103 | 6.0 | 151 | 5.4 | 254 | 5.6 |
| All | 375 | 2.5 | 478 | 3.0 | 853 | 2.8 |

[a]Average annual incidence (cases per 100,000 population).

Table 2. Annual incidence of early-onset and late-onset invasive group B streptococcus infections, Finland, 1995–2000

|  | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 |
|---|---|---|---|---|---|---|
| **Early-onset disease** | | | | | | |
| No. of cases | 37 | 37 | 34 | 38 | 33 | 32 |
| Incidence[a] | 0.6 | 0.6 | 0.6 | 0.7 | 0.6 | 0.6 |
| **Late-onset disease** | | | | | | |
| No. of cases | 8 | 16 | 6 | 10 | 9 | 7 |
| Incidence[a] | 0.1 | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |

[a]Cases per 1,000 live births.

All directors of the 38 hospitals with obstetric services responded. Written GBS prevention protocols existed in 30 (79%) hospitals. Most used a combination of risk-based and screening-based strategies; one routinely screened all pregnant women for prenatal GBS carriage. Recommendations for obstetric risk groups include screening patients for GBS when they have one of the following: premature delivery (87%), rupture of membranes without labor (82%), previous delivery of an infant with invasive GBS disease (79%), GBS bacteriuria (66%), and maternal fever during labor (53%). GBS specimens were usually obtained from the vagina (82%) or cervix (45%). No rectal cultures were taken. Culture was used to detect GBS in 82% of laboratories and antigen test in 34%. In 61% of the hospitals, chemoprophylaxis was given to all identified GBS carriers; the remaining 39% of hospitals required the presence of at least one additional obstetric risk factor before prescribing chemoprophylaxis (Table 3). When screening cultures were not performed or the results were not available at labor, chemoprophylaxis was most often given to risk groups with the following obstetric risks: intrapartum fever, previous delivery of an infant with invasive GBS disease, or prolonged rupture of membranes. Intrapartum chemoprophylaxis was given parenterally in 87% of hospitals and orally in 11%. Penicillin was recommended in 69%, cephalosporins in 19%, and aminopenicillins in 11% of hospitals.

## Discussion

Compared with rates previously reported from European countries, the incidence of early-onset GBS disease in Finland is relatively low (7–14). However, the incidence is twice as high as rates reported among white infants in the United States (6). Data from the study hospitals in Finland also indicate that most mothers of infants with early-onset disease did not receive intrapartum antibiotics.

In Europe, most studies documenting the occurrence of early-onset GBS disease during the past decade involved a single hospital (10,12–14). European population-based data from Norway in 2001 showed an incidence of 1 case per 1,000 live births (8). During the period of our surveillance, the incidence of early-onset and late-onset infection in Finland remained unchanged and comparable to rates in a previous nationwide study conducted from 1985 to 1994 (early-onset disease 0.62/1,000 live births; late-onset disease 0.13/1,000 live births) (15). The annual number of cases of early-onset disease appears low, but surveillance is limited to culture-confirmed cases of invasive disease. The number of newborns in whom GBS is treated empirically may therefore be larger.

We also identified considerable variation in rates of early-onset infection by health district. In Finland, the need for effective preventive measures was already emphasized during the 1980s, when GBS was identified as the most important etiologic agent of neonatal septicemia (16). The efficacy of intrapartum chemoprophylaxis has also been demonstrated by a Finnish study (17). However, the prophylaxis was only introduced to heavily colonized patients detected by the streptolatex test.

In our review of 26 cases of early-onset GBS disease, 1 case-patient received intrapartum antibiotics; 31% were screened prenatally for GBS, and 35% had a risk factor evident at the time of labor. Most case-patients were not screened and had no risk factors at the time of labor; of those not screened, four later developed a risk factor. Screening was performed for those in risk groups; some patients, such as those who had previously delivered infants with GBS disease or who tested positive for GBS bacteriuria during pregnancy, were unnecessarily screened. In addition, the site where cultures were taken and isolation method used differed from those recommended (5,18–22).

In the United States, the decline in the incidence of GBS disease in newborns coincided with the implementation of consensus guidelines for the prevention of perinatal GBS disease

Table 3. High risk groups for whom intrapartum antibiotic prophylaxis is recommended in 38 Finnish hospitals with obstetric services, 2002[a]

| Risk group | No. of hospitals (%) | |
|---|---|---|
|  | GBS specimen taken (result positive)[b] | GBS specimen not taken (results unknown) |
| GBS-positive mothers | 23 (61) | – |
| GBS bacteriuria during current pregnancy | 15 (39) | 15 (39) |
| Invasive GBS disease in previously delivered child | 25 (66) | 25 (66) |
| Delivery <37 wk gestation | 18 (47) | 9 (24) |
| Rupture of membranes ≥18 h | 26 (68) | 19 (50) |
| Intrapartum fever >38°C | 31 (82) | 33 (87) |

[a]GBS, group B streptococcus.

[b]Only one hospital routinely screened all pregnant women for prenatal GBS carriage.

beginning in 1996 (1). From 1993 to1998, the incidence of GBS declined 65% from 1.7 to 0.6 per 1,000 live births. Data from 1998 to 1999 indicate a further decline in incidence in selected surveillance areas. Among certain demographic groups, such as white infants, the rate has declined to 0.3 per 1,000 live births (6). A recent review of >300 cases of early-onset infection from the United States also showed missed opportunities for prevention, including cases that would not have been prevented even with perfect implementation of prophylaxis strategies: 21% of cases occurred despite administration of intrapartum antibiotics, 35% of case-patients had been screened perinatally for GBS, and 44% had a risk factor evident at the time labor (2,6). Of case-patients not screened for GBS, 40% showed none of the risk-based criteria for prophylaxis.

GBS infection in adults in Finland accounted for 67% of the total cases. The average annualized incidence of invasive GBS infection in adults varied from 1.6 to 5.6 per 100,000 population by age group. Although the incidence among elderly women was slightly lower than in elderly men, this number appears to be increasing. This finding is similar to previous population-based incidence data reported in 1989 to 1990 from metropolitan Atlanta, Georgia, where 48% of the total GBS cases were in adults; the annual incidence was 6.2 per 100,000 (3). Because the Atlanta study focused on nonpregnant adults and our study did not have information on the pregnancy status of the patients, age-specific rates cannot be compared. Recent U.S. data that included pregnant and nonpregnant adults indicate a marked increase in rates among adults, particularly in elderly persons and those with underlying illness, and vary between 2.1 to 21.9 per 100,000 by age group (4). The reasons for differences in rates of GBS disease between countries may include demographic differences, socioeconomic factors, and variations in clinical practices, such as the frequency of taking blood cultures in diagnostic examinations. Another suggested independent risk factor for both early-onset and late-onset GBS infection in neonates is being of black race (3,4). However, the association may also be linked to socioeconomic factors. The increasing prevalence of diabetes mellitus and other underlying conditions may contribute to the increasing rates of GBS infection in adults.

A common concern in the risk-based prevention approach is that a large number of women would receive unnecessary antibiotics. Widespread use of antibiotics can lead to an increase in allergic reactions, emergence of resistant strains, and cases of antibiotic colitis. The use of intrapartum antibiotics in the United States has doubled from 1996 to 1999, coinciding with GBS prevention implementation (23). We were unable to obtain information on how widely prophylaxis is currently used in Finland because data on type, dose, and time of administration of intrapartum antibiotics are not documented in hospital databases. Unnecessary antibiotic use could be reduced by not offering GBS prophylaxis to women who are not carriers. A recent study suggested that screening may be more effective in prevention than the risk-based approach (23). Screening reaches a broader population, and persons who are

screened are more likely to receive prophylactic antibiotics. However, wide-scale screening for GBS colonization may be difficult to implement.

The results of our study should be used to develop and implement national guidelines for prevention of perinatal GBS. Such guidelines would standardize prevention practices, rationalize the use of intrapartum antibiotics, and reduce the incidence of perinatal GBS disease. Further studies should be done to investigate the reasons for incidence increase among elderly women.

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Address for correspondence: Outi Lyytikäinen, Department of Infectious Disease, Epidemiology, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland; fax: 358-9-47448468; email: outi.lyytikainen@ktl.fi

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# Alfalfa Seed Decontamination in *Salmonella* Outbreak

Christopher J. Gill,*† William E. Keene,* Janet C. Mohle-Boetani,‡ Jeff A. Farrar,§ Patti L. Waller,¶
Christine G. Hahn,# and Paul R. Cieslak*

Based on in vitro data, the U.S. Food and Drug Administration recommends chemical disinfection of raw sprout seeds to reduce enteric pathogens contaminating the seed coats. However, little is known about the effectiveness of decontamination at preventing human disease. In 1999, an outbreak of *Salmonella enterica* serotype Mbandaka occurred in Oregon, Washington, Idaho, and California. Based on epidemiologic and pulsed-field gel electrophoresis evidence from 87 confirmed cases, the outbreak was linked to contaminated alfalfa seeds grown in California's Imperial Valley. Trace-back and trace-forward investigations identified a single lot of seeds used by five sprout growers during the outbreak period. Cases of salmonellosis were linked with two sprout growers who had not employed chemical disinfection; no cases were linked to three sprout growers who used disinfection. This natural experiment provides empiric evidence that chemical disinfection can reduce the human risk for disease posed by contaminated seed sprouts.

Despite the popular perception of sprouts as health food, alfalfa and other varieties of raw seed sprouts are common vehicles for produce-associated bacterial foodborne illness. Although sprout-associated outbreaks have been reported since 1973 (1), back-to-back multinational outbreaks of gastroenteritis with *Salmonella enterica* serotypes Newport and Stanley in 1995 and 1996 (2,3) and the 1996 Sakai city outbreak of enterohemorrhagic *Escherichia coli* O157:H7 in >5,000 Japanese schoolchildren have refocused attention on the public health hazard posed by seed sprouts (4).

The risk for disease from sprouts is connected to seed production and distribution factors and the sprouting process itself. Seeds may be reared, harvested, milled, and sprouted locally or shipped globally to sprout growers; bacterial contamination may occur at any point in this chain (5). During germination, seeds are presoaked in water and then germinated in a warm, moist, aerated environment for 3 to 7 days. Replication of pathogens by three to five orders of magnitude may occur dur-

ing sprouting, resulting in high pathogen levels on mature sprouts, despite the fact that initial densities are low and the pathogens dispersed irregularly throughout seeds (6). In an experimental model of seed contamination, *Salmonella* Stanley added to alfalfa seeds increased from ~$2 \times 10^3$ bacteria per gram of mature sprouts to $10^7$ bacteria per gram after 48–72 hours incubation, without affecting the appearance, smell, or taste of the sprouts (6).

Methods proposed to reduce the risk to human consumers include testing seeds, irrigation water, and sprouts for pathogens (7–12) and disinfecting seeds (13–16). In vitro, treating seeds with 20,000 ppm calcium hypochlorite [$Ca(OCl)_2$] pregermination reduces pathogen densities by up to 2.2 logs (13,14). Higher concentrations of disinfectant or the use of concentrated acids, high temperatures, or bleaches reduces pathogen levels by >3 log; these treatments substantially reduce the proportion of seeds that germinate (16,17). Notably, none of these methods completely eliminates pathogens on seeds. Based on these data, the U.S. Food and Drug Administration (FDA) currently recommends that seeds be treated with 20,000 ppm $Ca(OCl)_2$ pregermination and that sprouts and spent irrigation water samples be periodically tested for enteric pathogens. The number of sprout growers (referred to as "sprouters") who follow these guidelines, as well as the proportion of sprouts grown from disinfected seeds, is unknown.

*S.* Mbandaka, an uncommon serotype in Oregon, occurred from 1988 to 1998 at an average rate of 1.5 cases per year. From January to April 1999, the Oregon Department of Human Services (ODHS) conducted an outbreak investigation in response to a sharp increase in cases of *S.* Mbandaka in what soon proved to be a multistate outbreak. This investigation ultimately provided insight into the efficacy of seed disinfection.

## Methods

We defined case-patients as persons with culture-confirmed *S.* Mbandaka infection and onset of acute illness from January 1 to April 15, 1999. Cases were excluded if they were subsequently shown to have a pulsed field gel electrophoresis (PFGE) pattern that differed from the outbreak pattern. If the illness onset date was unknown, the specimen collection date minus 2 days was used instead.

To generate hypotheses about potential exposure vehicles, ODHS conducted open-ended interviews with the first 10

*Oregon Department of Human Services, Portland, Oregon, USA; †Tufts University-New England Medical Center, Boston, Massachusetts, USA; ‡California Department of Health Services, Berkeley, California, USA; §California Department of Health Services, Sacramento, California, USA; ¶Washington State Department of Health, Shoreline, Washington, USA; and #Idaho Department of Health and Welfare, Boise, Idaho, USA

Oregon case-patients. Based on these interviews, we performed a 2:1 (controls:cases) age-matched case-control study using these cases. Age-matched controls were identified by sequentially adding or subtracting one digit from each case-patient's telephone number prefix. Matched ranges were: 1–18, 19–50, and 51–100 years of age. Using a standardized food questionnaire, we asked respondents about consumption of food that typically carries *Salmonella* during the preceding week.

After alfalfa sprouts were implicated, we traced the source of sprouts from the case-patients to the brand of sprout's point of production to identify a common source for the contaminated sprouts. Onsite investigations of an implicated sprouter were conducted in partnership with the Washington Department of Agriculture and the FDA. Seed, water, and sprout samples were collected for culture with sterile swabs and containers.

Because alfalfa seeds are often produced in bulk, we conducted trace-back and trace-forward investigations using purchase invoices and shipping records to identify other sprouters who may have purchased and used potentially contaminated seeds from the same production lot. Other sprouters who purchased this lot were asked about their use of the implicated lot, including quantities sprouted and their seed-disinfection practices. To identify additional cases, we queried health departments throughout the United States and Canada, with specific attention to states where seed from the implicated lot had been distributed.

### Laboratory Methods

All *Salmonella* isolates from patients were serotyped at the Oregon, California, Washington, and Idaho state laboratories. *Salmonella* cultures were performed according to standard methods (18,19). PFGE, by using the restriction enzyme *Xba*I (Boehringer Mannheim, Indianapolis, IN), was used to corroborate genetic relatedness of *S.* Mbandaka isolates according to the method of Tenover et al. (20). Given the rarity of *S.* Mbandaka, we did not consider it necessary to use two or more restriction enzymes to achieve a still higher degree of resolution with PFGE. Two *S.* Mbandaka isolates from 1997 to 1998 were used as reference standards.

### Statistical Methods

We calculated matched odds ratios, 95% confidence intervals, and p values using Exact (21). A two-tailed p value of <0.05 was considered significant. Using food frequency data from Oregon FoodNet showing that ~10% of Oregonians consume sprouts on a weekly basis (22), we performed a binomial calculation of the probability that the observed proportion of sprout exposure among cases was due to chance (23).

### Results

#### Descriptive Epidemiology

From January 1 to April 15, 1999, a total of 89 cases of *S.* Mbandaka were identified in four states: Oregon (42 cases), Washington (19 cases), Idaho (7 cases), and California (21

cases) (Figure 1); 74% of the case-patients were women, and the median age was 28 years of age (range, 6 months to 98 years; interquartile range: 19–41 years). Two additional cases were not included because neither could be linked epidemiologically with the outbreak and both had different PFGE patterns. *S.* Mbandaka was cultured from 69 stool samples, 14 urine samples, three blood cultures, one abscess, one blood and stool sample, and one unspecified source. The association between female sex and urinary isolates tended towards significance (p=0.06, Fisher exact test). Two cases clustered within a household, but were not considered to represent secondary spread of the disease, as they occurred concurrently. No patients died.

Nine of the first 10 case-patients and none of 20 matched control-patients reported eating or handling alfalfa sprouts (matched odds ratios were undefined [zero in denominator]; 95% confidence interval 3.4 to ∞; p<0.001). No other food vehicles were statistically associated with illness. From the binomial theorem, given an expected frequency of 10%, the probability of 9 out of 10 persons reporting sprout consumption within the past week was <1/1000 (22).

#### Identification of Sprout Source

The first 10 Oregon case-patients reported eating sprouts from one or more of five growers (sprouters A, B, C, D, and X). Trace-back investigation showed that the common link between most cases was sprouter X (Figure 2). Eight of nine case-patients reported eating sprouts that could have come from sprouter X. Five of these eight cases had potential links to
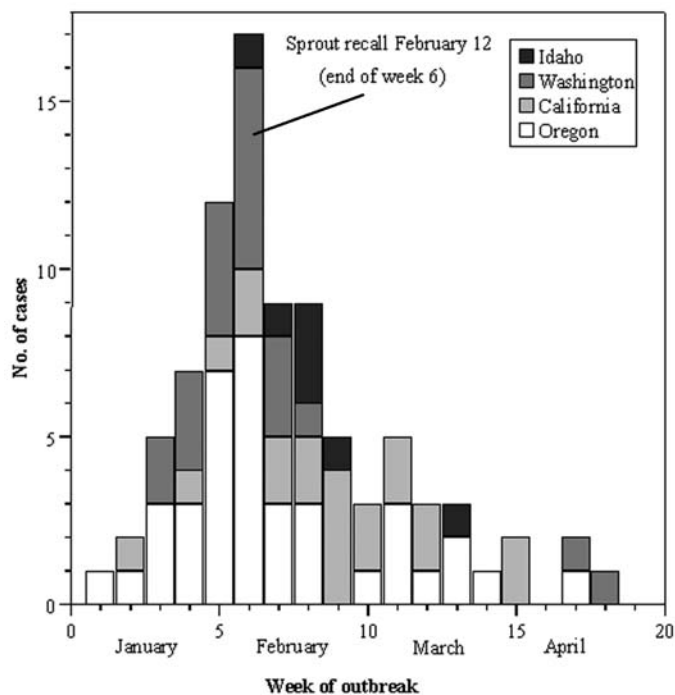


Figure 1. Epidemic curve of *Salmonella* Mbandaka outbreak, 1999. Line indicates the timing of the Oregon Health Division's press release alerting the public of the outbreak. A lot L seed embargo and voluntary recall of brand X sprouts also occurred at this time.
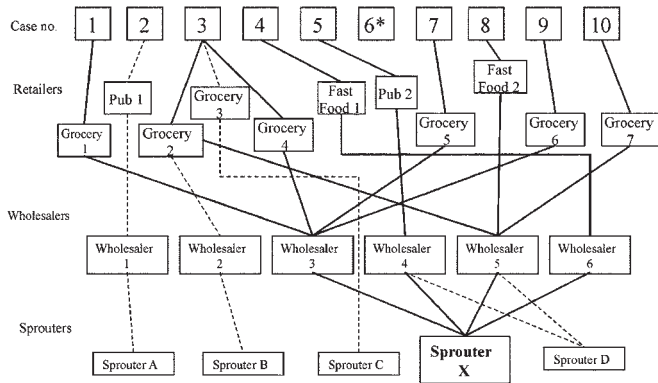
Figure 2. Trace-back investigations of Oregon cases of *Salmonella* Mbandaka. *Case-patient 6 recalled no sprout exposure. Solid lines indicate trace-back routes leading to sprouter X. Dashed lines indicate trace-back routes leading to other sprouters.

other sprouters in addition to sprouter X, while three of the eight cases could be traced only to sprouter X.

The index patient of *S.* Mbandaka provided a leftover sample of sprout X brand alfalfa sprouts. Cultures from this sample subsequently grew *S.* Mbandaka.

## Onsite Investigations at Sprouter X's Facility

Based on these investigations, representatives of the Washington Department of Health and FDA inspected the sprouter X facility on February 12, 1999, and determined that alfalfa sprouts grown during the outbreak period came from a single seed lot (lot L). Trace-forward information from the seed distributor and interviews with the other area sprout growers confirmed that sprouter X was the only grower in the Pacific Northwest to have purchased lot L seeds. Moreover, the illness onset date for the Oregon index case occurred 2 weeks after this sprouter received a shipment of lot L and 1 week after sprouts grown from lot L first reached market.

*S.* Mbandaka was isolated from 11 of 12 alfalfa sprout samples, and one of two lot L seed samples obtained from the sprouter X facility. No pathogens were identified from this sprouter's samples of radish, onion, and clover sprouts, seed samples from non–lot L seeds, or samples of irrigation water.

Inspectors determined that no seed disinfection had been used at the sprouter X facility. The owner of the facility informed us that disinfection was "unnecessary" because seed samples had tested negative for pathogens before being shipped from the seed broker.

After this inspection, sprouter X issued a voluntary recall of all products. Existing stocks of alfalfa sprouts were impounded, and the company was instructed to cease further production of sprouts using lot L seeds. A press release from the Oregon Department of Human Services was issued on February 12, 1999, advising consumers to return brand X sprouts and retailers to return unsold stocks.

## Genetic Analysis of *S.* Mbandaka Isolates

The PFGE patterns for the *S.* Mbandaka isolates from stool samples from the initial 10 case-patients, the partially consumed box of sprout X retrieved from case-patient 1, and the onsite sprout and seed sample cultures were indistinguishable but clearly differed from the two *S.* Mbandaka reference samples obtained in 1997 and 1998. Of the final 89 cases, 87 had identical PFGE patterns; 2 case-patients included in the total were not tested for technical reasons.

## Trace-Back and Trace-Forward Investigations of Lot L

Sprouter X purchased lot L seeds from a California broker. This broker, in turn, purchased seeds from California seed producer Z. Purchase invoices indicated that 5,454 kg of seed from 18,000 kg of lot L had been distributed among five sprout growers other than sprouter X: sprouters E, F, G, H, and Y (Table). Four sprouters were located in California and one in Florida (sprouter E). Of the 5,454 kg of seed sold to sprouters, 3,864 kg (70.2%) had already been sprouted by the time of the embargo. Of this seed, sprouters X and Y had used 2,610 kg (68%) and sprouters E, F, and G had used 1,254 kg (32%) (Table). Sprouter H had not yet used any lot L seed.

Of the five sprouters who used lot L seeds, two were linked to the *S.* Mbandaka outbreak. Isolates from Washington, Oregon, and Idaho were traced to sprouter X in Washington State; all California cases were traced to sprouter Y. Neither sprouter X nor Y had used seed disinfection during the outbreak period. Of the three sprouters (E, F, and G) using lot L seed but not linked to cases of *S.* Mbandaka, all reported disinfecting their seeds with 2,000–20,000 ppm $Ca(OCl)_2$, or 200 ppm sodium hypochlorite (Table).

All seeds from lot L came from a single farm in California's Imperial Valley. Although we were unable to determine how the seeds became contaminated, an inspection of the farm showed numerous opportunities for contamination by wild and domestic animals or river water.

Table. Fate of lot L alfalfa seeds

| Sprouter (state) | Quantity purchased (kg) | Quantity sprouted (kg) | Seed disinfection | Linked cases |
|---|---|---|---|---|
| Sprouter X (Washington) | 909 | 860 | None | 68 |
| Sprouter Y (California) | 1,818 | 1,750 | None | 21 |
| Sprouter E (Florida) | 909 | 909 | 20,000 ppm $Ca(OCl)_2$ | 0 |
| Sprouter F (California) | 909 | 45 | 20,000 ppm $Ca(OCl)_2$ | 0 |
| Sprouter G (California) | 450 | 300 | 500 ppm NaOCl | 0 |
| Sprouter H (California) | 450 | 0 | ND[a] | 0 |
| Totals | 5,454 | 3,864 | | |

[a]ND, not determined. The method of disinfection did not apply to sprouter H because this sprouter had not used any lot L seeds by the time of the embargo.

## Discussion

The epidemiologic, laboratory, and environmental evidence shows that alfalfa sprouts germinated from lot L seeds were the cause of this outbreak. The fact that two geographically distant sprouters (X and Y) were linked to cases of *S*. Mbandaka suggests that the seeds became contaminated before distribution to sprouters. Given the historic underreporting of salmonellosis, the 89 confirmed cases of *S*. Mbandaka probably represent several thousand cases (24). Salmonellosis is typically most prevalent in patients <1 year of age (25,26), so the fact that women 19–41 years of age predominated in this outbreak is noteworthy. This pattern was observed in several previous sprout-associated outbreaks (3,27–30). We believe this pattern reflects the demographics of sprout consumers and does not imply that women of this age range are inherently predisposed to salmonellosis in general or to *S*. Mbandaka in particular. As noted in previous outbreaks, the urinary tract was a common site of infection among women (28).

The outbreak was limited to consumers of sprouts grown by two sprouters who used the same seed lot. Neither sprouter used the FDA-recommended seed-disinfection process. Of the three sprouters who disinfected their seeds, none were linked to cases, although they had used 32% of the lot L seed that was sprouted. This finding suggests that seed disinfection does reduce the risk for human disease posed by seed sprouts.

Since a formal randomized trial of seed disinfection in the community would be unethical, our understanding of the usefulness of disinfection is based largely on observations made in the course of outbreak investigations. In the current study, this outbreak fortuitously served as a natural experiment of the effectiveness of seed disinfection.

Few other published outbreak investigations have addressed the value of disinfection at preventing human disease. Clover sprouts were implicated in a 1999 outbreak in Colorado (31). The two sprouters involved also grew sprouts from a common seed lot, though only one sprouter reported followed the FDA's disinfection guidelines. Salmonellosis linked to the sprouter using disinfection occurred at a rate of 0.29 cases per 50 kg bag of seed versus 1.13 cases per 50 kg bag from the sprouter not using disinfection. The investigators concluded that disinfection reduced, but did not eliminate, the risk for human salmonellosis.

In another recent outbreak, Proctor et al. investigated a multistate alfalfa sprout–associated salmonellosis outbreak in which 20,000 ppm $Ca(OCl)_2$ disinfection was used (30). This outbreak was confined to a single sprouter; in the absence of a "control" sprouter not using disinfection, ascertaining whether disease rates would have been even higher is impossible. Nevertheless, both outbreaks amply demonstrate that disinfection is an imperfect remedy for the problem of contaminated seed sprouts.

The inoculum size needed to cause infection is known to vary between different serotypes of *Salmonella* (32–34). Because disinfection reduces but does not eliminate pathogens, this variation might account for some of the apparent disinfection failures if levels were not reduced below some critical threshold. Human error may also be to blame. In a recent survey of disinfection practices of area mung-bean and green sprouters (green sprouts include alfalfa, clover, radish, and onion sprouts) who reported observing the FDA guidelines, California investigators noted that six of seven green sprouters and one of nine mung-bean sprouters succeeded in reaching the target of 20,000 ppm $Ca(OCl)_2$ (J. Thomas, unpub. data). Accordingly, future investigations should include careful reviews of the disinfection records of implicated sprouters. Receipts for seed-disinfection chemicals, protocols for disinfection, and disinfection logs may prove useful clues to actual disinfection practices. Asking the sprouter to simulate the disinfection process may also be instructive.

Given these limitations, a more reliable method of decontamination is needed if eating sprouts is to be safe. Gamma irradiation of sprout seeds or mature product reduces or eliminates colonizing bacteria. However, the prohibitive expense of irradiating machinery places this technology beyond the means of most sprouters. Moreover, irradiating seeds reduces the germination yield and affects the appearance of mature sprouts that germinate (35,36). Marketing irradiated sprouts to health-conscious consumers may prove challenging, given the frequent misconception that irradiated sprouts are themselves radioactive. A concerted public awareness campaign might avoid consumer anxiety about this process. Sprouters could also pool resources towards the collective purchase or use of irradiating machines. Cooking sprouts is highly effective at eliminating pathogens but is not a popular remedy because sprouts are typically consumed raw.

The pathogen detection assay used at the request of sprouter X did not detect the *S*. Mbandaka contaminating the lot L seeds. The test used a proprietary protocol wherein several grams of seed were randomly sampled for testing from each 50-kg bag of seed by using a monoclonal antibody-based enzyme-linked immunoassay (Silliker Laboratories, pers. comm.). Regardless of the assay's sensitivity, this technique appears highly vulnerable to sampling error unless the pathogen is uniformly distributed throughout the seed. Notably, research to date indicates that pathogens are dispersed heterogeneously and at low densities on seeds (37,38). Compounding this vulnerability, the assay used was designed only to detect *Salmonella*, a shortcoming given that enterohemorrhagic *E. coli* outbreaks linked to sprouts have occurred repeatedly (28,39–43).

In the current outbreak, a negative test was cited by the sprouter X's proprietor as the rationale for disregarding FDA recommendations. This failure is noteworthy given that *S*. Mbandaka was isolated from seeds both by the California Microbial Diseases Laboratory, Berkeley, California, and the FDA Laboratory in Bothell, Washington., by using more sensitive enrichment and detection processes. Currently, FDA does not advocate routine seed screening, although it does recommend testing sprouts or spent irrigation water samples for enteric pathogens (18), neither of which was performed by sprouter X.

For the time being, FDA should continue to promote adherence with its guidelines for disinfecting seeds and testing

sprouts and irrigation water. The International Sprout Grower's Association (ISGA), a private trade group, has responsibly encouraged adherence among its members by allowing sprouters who use 20,000-ppm Ca(OCl)$_2$ disinfection and pass a third-party production inspection to label their sprouts as ISGA-certified. However, this precaution has yet to be translated into a marketing advantage through retailer and consumer education; compliance remains voluntary, and no systematic data exist indicating the proportion of sprouters who adhere to the guidelines. Adherence with a uniform disinfection process might be improved if made subject to regulation or if retailers were to insist on "ISGA-certified treated seed" in their purchase contracts for sprouts.

Given repeated outbreaks, seed testing that is falsely reassuring, ineffectiveness and incompleteness of seed disinfection, and a lack of commitment to disinfected product from retailers, we think that consistently rendering raw sprouts free of enteric pathogens is not practical. Persons at increased risk for invasive salmonellosis and those wishing to reduce their risk for foodborne infections may be well advised to avoid sprouts entirely.

Dr. Gill is assistant professor of international health at the Center for International Health and Development at Boston University's School of Public Health. His research interests include HIV/AIDS, malaria, diarrheal illness, and respiratory diseases in children living in resource-poor countries.

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Address for correspondence: Dr. William E. Keene, Acute and Communicable Disease Prevention, Oregon Dept. of Human Services, 800 NE Oregon St., Ste. 772, Portland, OR 97232, fax: 503-731-4798; email: william.e.keene @state.or.us

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# Scrub Typhus in the Torres Strait Islands of North Queensland, Australia

Antony G. Faa,* William J.H. McBride,†
Gaynor Garstone,‡ Robert E. Thompson,*
and Peter Holt*

Scrub typhus, caused by *Orientia tsutsugamushi*, occurs throughout Southeast Asia. We descript ten cases that occurred in the Torres Strait islands of northern Australia during 2000 and 2001. Preceding heavy rain may have contributed to the outbreak. The successful use of azithromycin in two pediatric patients is also reported.

Scrub typhus is a rickettsial disease caused by the bacteria *Orientia tsutsugamushi* and is transmitted to humans from the bite of a larval trombiculid mite. The disease is distributed throughout Southeast Asia and is restricted by the distribution of the host mite (1). Scrub typhus has been recognized as being endemic to north Queensland since the 1920s (2). More recently, the disease has been recognized in the Northern Territory (3) and the northwest of western Australia (4). In north Queensland the disease is most commonly recognized between Ingham and Cooktown. In a recent review of cases from north Queensland, none were reported outside of this area (5). In March 2000, scrub typhus was diagnosed in a visitor to Torres Strait from Papua New Guinea. Additional cases were subsequently diagnosed in residents of Torres Strait. We describe a cluster of cases from Darnley Island in the Torres Strait. This island, and possibly others in the Torres Strait, should now be considered as scrub typhus–endemic foci.

## Case Studies

A 35-year-old man, a visitor from Papua New Guinea, arrived at the Darnley Island Health Centre in March 2000, complaining of headache and symptoms of fever. He had arrived on Darnley Island 24 days before onset of illness. He began a course of oral quinine for suspected malaria but remained ill and was transferred to Thursday Island Hospital. He was noted to be febrile (40.2°C) and had marked conjunctival injection. No obvious focus of infection was evident. The day after admission he was still febrile and was given Timentin (ticarcillin plus clavulinic acid), gentamicin, and doxycycline. His condition gradually improved, and the fever subsided in 5 days. Scrub typhus serologic test results were strongly positive (Table, patient 1).

A 19-year-old patient (patient 2) was transferred from

*Thursday Island Hospital, Queensland, Australia; †Cairns Base Hospital, Queensland, Australia; and ‡Darnley Island Health Centre, Queensland, Australia

Murray Island to the Thursday Island Hospital in May 2000. He had been ill for 12 days with fever, frontal headache, and intermittent vomiting. He had a documented temperature of 40.1°C before admission to hospital and had been treated symptomatically with aspirin and metoclopromide. He had not left Murray Island for several months and had never traveled to Papua New Guinea. He denied knowledge of any mite bites. On admission he looked ill and complained of lethargy; his temperature was 39.4°C. He had slight conjunctival injection and mild tenderness in the right upper quadrant of his abdomen. A typical black eschar approximately 0.5 cm in diameter was found on his upper right thigh with associated tender right inguinal lymphadenopathy. Tests indicated a normal leukocyte count (9.8 x $10^9$/L), thrombocytopenia (platelet count 79,000/μL), increased creatinine (0.14 mmol/L), and abnormal liver function tests (γ-glutamyl transferase 55 U/L, normal <50; alanine aminotransferase 280 U/L, normal <45; aspartate aminotransferase 223 U/L, normal <40). He received doxycyline, became afebrile after 48 hours, and made an uncomplicated recovery

In August 2000, a 29-year-old man (patient 3) arrived at the primary health center on Thursday Island with a 5-day history of fevers, sweats, lethargy, and headache. As a field worker with the local electricity authority, he had visited several of the outer Torres Strait islands (Yorke, Darnley, Mabuaig, Yam, Badu, and Stephen) in the 3 weeks before becoming ill. These visits included a 3-day trip to Darnley Island 2 weeks before his fevers began. Some of his work involved clearing vegetation from the fence lines of the local power plants. On examination he looked ill, but no abnormal physical signs were found. He did not have an eschar or rash. He was seen 5 days later and still had a temperature of 40°C. He was not treated with antibiotics, and his fever settled spontaneously during the 3 weeks.

A 28-year-old woman (patient 4) arrived at Darnley Island Health Centre in January 2001 with her 9-year-old son (patient 5); both complained of being ill for approximately 1 week. They reported fevers, headache, and generalized myalgia. The child also complained of vomiting and was noted to have a dry cough with some rhinorrhea. He was started on a course of amoxicillin. The next day, when seen at the visiting doctors clinic, both mother and son were given oral quinine to treat possible malaria. Over the next few days, both remained febrile with temperatures up to 40°C. They were transferred to Thursday Island Hospital. The mother had a typical black eschar on her left breast (Figure 1) with associated regional lymphadenopathy. She also had conjunctival injection. Oral doxycycline was administered, and the fever resolved over 3 days. Her son was treated with azithromycin (250 mg a day for 3 days) and became afebrile within 24 hours.

In the next 3 weeks, four more patients (one adult [patient 7], two teenagers [patients 6 and 8], and one 5-year-old child [patient 9]) arrived at Darnley Island Health Centre with nonspecific febrile illnesses associated with headache. Two had eschars (one located on the scrotum and another on the scalp) with associated regional lymphadenopathy. One patient had conjunctival injection, and another had a fine truncal rash. Two

Table. Demographic, clinical and serologic data for patients with scrub typhus, Torres Strait islands, Queensland, Australia

| Patient no. | Age (y)/sex | Date | Island | Eschar | *Orientia tsutsugamushi* antibody titer[a] (d after onset) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0–7 | 8–14 | 15–21 | 22–28 | >29 |
| 1 | 35/male | Mar 2000 | Darnley | No | | 8,192 | | | 8,192 |
| 2 | 19/male | May 2000 | Murray | Yes | <64 | 4,096 | | | 4,096 |
| 3 | 29/male[b] | Aug 2000 | — | No | 1,024 | 1,024 | | | 1,024 |
| 4 | 28/female | Jan 2001 | Darnley | Yes | | NR | | 512 | |
| 5 | 9/male | Jan 2001 | Darnley | No | | | 256 | | 256 |
| 6 | 14/female | Jan 2001 | Darnley | No | | 512 | 512 | | |
| 7 | 36/male | Jan 2001 | Darnley | Yes | NR | | 512 | | |
| 8 | 19/male | Jan 2001 | Darnley | No | 64[c] | | | 256 | |
| 9 | 5/male | Feb 2001 | Darnley | Yes | | >1,024 | >1,024 | | |
| 10 | 30/male[b] | Apr 2001 | — | Yes | NR[c] | 128 | | | |

[a]Serologic tests were performed by using an immunofluorescent antibody method; NR, nonreactive.
[b]Patient visited several islands in 3 weeks before becoming ill.
[c]Polymerase chain reaction for scrub typhus positive (by using primers for 56-kDa antigen gene).

were treated with oral doxycycline and became well soon after starting treatment. Patient 6 was successfully treated with azithromycin (1,000 mg as a single dose). Patient 9 had been given a course of oral and intramuscular penicillin before scrub typhus was suspected and remained ill with fevers and abdominal pain (with right upper quadrant tenderness). He was transferred to the hospital 2 weeks after being initially seen but was afebrile on admission and remained well with no additional treatment. An additional case of scrub typhus (patient 10) was documented in April 2001.

**Epidemiologic Findings and Community Interventions**

After recognition of this cluster of scrub typhus cases, a doctor and indigenous health worker traveled to Darnley Island to interview patients and educate the local community about the disease. The six patients had not left Darnley Island in the 6 weeks before becoming ill. The patients lived on different parts of the island, and no common area was visited before illness onset. Patient 4 was an exception and had harvested mangoes in an area of jungle close to her house where her son (patient 5) played. The transmission of scrub typhus on Darnley Island probably occurred in the densely vegetated areas close to most domestic dwellings on the island. A public meeting was held, which focused on increasing the general awareness of the signs and symptoms of scrub typhus and the importance of early diagnosis and treatment. The meeting included local indigenous health workers. Aspects of personal protection against mite bites were also stressed, including wearing protective boots and trousers and using insect and mite repellants when going into potential areas of transmission.

**Conclusions**

The Torres Strait islands (Figure 2) lie between Cape York Peninsula in Queensland and Papua New Guinea. The islands have a population of approximately 9,000 mainly indigenous inhabitants. Approximately half of the people live on or near Thursday Island, which is the main commercial and government center. A 38-bed general hospital is located there. The other half of the population lives on the 15 outer island communities that stretch between the Australian and Papua New Guinea mainland. Health centers are located on each of these islands and are staffed by remote area nurses, indigenous healthcare workers, or both.

The cases of scrub typhus infection we describe are not the first to be described from the Torres Strait islands. Scrub typhus was previously reported in the 1950s, with a single case from Hammond Island (near Thursday Island), and another in a diver from a lugger "near Darnley Island" (2).

We describe at least two islands where transmission of scrub typhus occurred in the Torres Strait during the wet seasons of 2000 and 2001. One infection occurred on Murray Island, and seven occurred on Darnley Island. Another patient lived on Thursday Island but traveled to several outer islands in the weeks before becoming ill and probably acquired his infection on Darnley Island. The occurrence of six cases of scrub typhus on Darnley Island over a 3-week period is important, representing the infection of nearly 2% of the total population (375) (6). Rainfall for the region was well above average in



Figure 1. Eschar on the breast of a patient with scrub typhus during an outbreak on Darnley Island, Torres Strait.
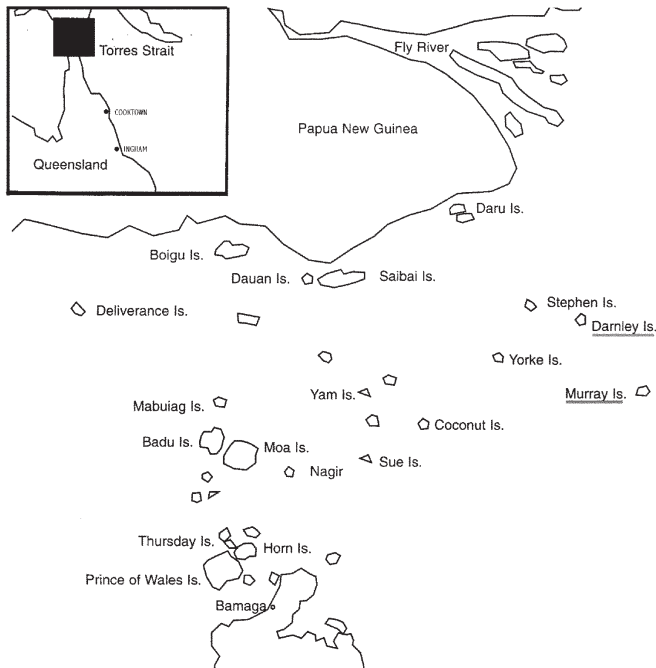
Figure 2. Map of Torres Strait islands showing areas of scrub typhus transmission, Darnley and Murray Islands, Torres Strait, 2000–2001.

November and December 2000, the beginning of the wet season (7). The resulting dense vegetation would have provided favorable conditions for the transmission of disease. We believe that *O. tsutsugamushi* has probably existed in the region for many decades, rather than having been recently introduced, and climatic conditions favored the transmission to humans in the early part of 2001.

Our series included two children and one young teenager. A 9-year-old boy and 14-year-old girl responded well to treatment with azithromycin. Azithromycin has been used successfully in the treatment of scrub typhus in a small number of pregnant women (8), but to our knowledge these are the first published cases of its clinical use in children with the disease.

Dr. Faa is a general practitioner currently working at St. Mary's Hospital in Papua New Guinea. His interests include rural and remote medicine in indigenous populations and tropical medicine.

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Address for correspondence: W.J.H. McBride, Department of Pathology, Cairns Base Hospital, Cairns, Queensland, Australia; fax: 61 7 40506699; email: john_mcbride@health.qld.gov.au

**Instructions for Infectious Disease Authors**

**Dispatches**

Dispatch articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

# Early-Season Avian Deaths from West Nile Virus as Warnings of Human Infection

**Stephen C. Guptill,\* Kathleen G. Julian,†
Grant L. Campbell,‡ Susan D. Price,\*
and Anthony A. Marfin‡**

An analysis of 2001 and 2002 West Nile virus (WNV) surveillance data shows that counties that report WNV-infected dead birds early in the transmission season are more likely to report subsequent WNV disease cases in humans than are counties that do not report early WNV-infected dead birds.

West Nile virus (formal name: *West Nile virus* [WNV]) was first detected in the United States during an encephalitis outbreak in New York City in September 1999 (1). Since then, WNV activity has been reported from 42 additional states and the District of Columbia (2). Avian, equine, and human illnesses are most often reported. Analysis of surveillance data from 2001 and 2002 chronicles the spread of infection and may provide a means of locating areas where human illness is more likely to occur.

## The Study

Surveillance data have often been used in the study of arboviral disease outbreaks (3,4). ArboNET, a cooperative WNV surveillance program maintained by the Centers for Disease Control and Prevention and 48 states, five cities, and the District of Columbia, collects surveillance data on a continuous basis. These data include reports of WNV-infected mosquitoes, sentinel animals, dead birds, and ill humans and horses (5). In 2001, 328 counties reported a total of 7,333 WNV-infected dead birds (range per county: 1–350). The first WNV-infected dead bird was found on April 8, and the last was found on December 26. Sixty-six human cases of WNV disease were reported from 39 different counties in 10 states,[1] including two outpatient West Nile fever cases. No single county reported more than four human cases. Onset of illness was on July 13 for the first human case and December 7 for the last reported case. Of particular interest is the date that the first WNV-infected dead bird was found in a given county. These dates ranged from the week ending April 14 to the week ending December 8.

In this retrospective cohort study, all U.S. counties that reported dead WNV-infected birds were categorized on the basis of whether a WNV-infected bird was found early in the transmission season (i.e., before August 5) and whether at least one subsequent human disease case was reported from the county. A relative risk (RR) statistic was calculated as follows: The proportion of counties that reported human cases among the counties that found infected birds before August 5 was divided by the proportion of counties that reported human cases among the counties that did not find infected birds before August 5.

## Results

Of 93 counties that reported at least one WNV-infected bird before August 5, 28 (30%) subsequently reported a human WNV disease case in 2001 compared to 11 (4.7%) of 235 counties that did not report an infected bird (RR 6.43, 95% confidence interval [CI] 3.34 to 12.38). In other words, in 2001, counties that reported a WNV-infected dead bird before August 5 were more than six times more likely than other counties to report a human WNV disease case (Figure 1).

As Figure 1 shows, the 2001 outbreak had two distinct geographic foci, the Northeast and the Southeast United States. These areas were analyzed separately to determine if the correlation between WNV-positive birds and human cases was true in different ecologic regions. The Northeast region consisted of Maryland, New Jersey, Pennsylvania, New York, Connecticut, Rhode Island, and Massachusetts. The Southeast region consisted of Florida, Georgia, Alabama, and Louisiana. The Northeast region contained 22 of the 39 counties in which human cases occurred. The Southeast contained the remaining 17 counties. RR statistics were significant for both regions (Northeast: RR 11.57, 95% CI 3.58 to 47.99; Southeast: RR 2.38, 95% CI 0.89 to 6.39).

Recently, provisional totals for the 2002 WNV surveillance data have become available through ArboNET. Given the great
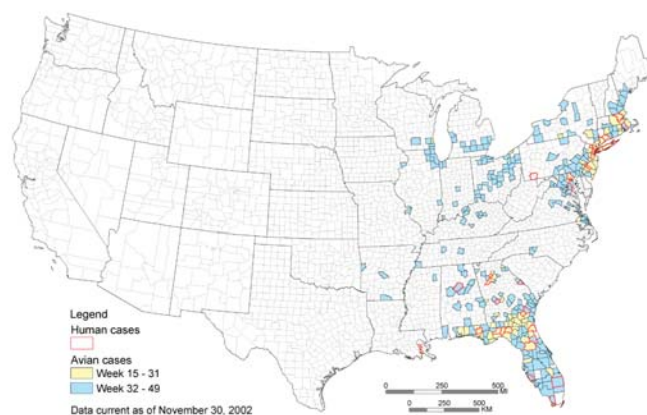


Figure 1. Counties reporting avian deaths and human illness caused by West Nile virus (WNV), January 1–December 31, 2001. Counties reporting human illness are outlined in red. The color within the county indicates the date when the first avian death from WNV was reported in that county. Counties that report dead birds early in the year (yellow) are more likely to report subsequent disease cases in humans.

\*U.S. Geological Survey, Reston, Virginia USA; †Hershey Medical Center, Hershey, Pennsylvania, USA; and ‡Centers for Disease Control and Prevention, Atlanta, Georgia

[1]These figures do not include a human case in New York that was not located to the county level.
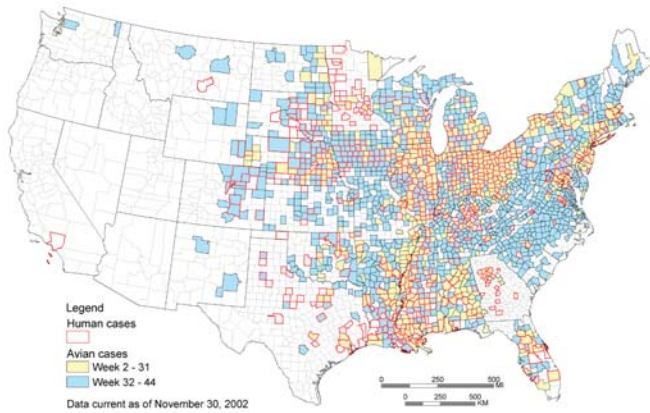
Figure 2. Counties reporting avian deaths and human meningitis/encephalitis caused by West Nile virus (WNV), January 1–November 30, 2002. Counties reporting human illness are outlined in red. The color within the county indicates the date when the first avian death from WNV was reported in that county. Counties that report dead birds early in the year are more likely to report subsequent disease cases in humans

increase in the geographic extent and the 50-fold increase in the number of human cases of WNV, we repeated this analysis by using the provisional data for 2002 to see if similar results would be obtained. A great deal of variation in the reporting of WNV fever cases has occurred between states. For this reason, only WNV meningitis and encephalitis cases were included in this analysis. In the provisional figures for 2002, a total of 504 counties reported human cases of WNV meningoencephalitis, and 1,719 counties reported WNV-infected birds. Of 632 counties that reported at least one WNV-infected bird before August 4, a total of 284 (45%) subsequently reported a human WNV disease in 2002 compared to 220 (19%) of 1,162 counties that did not report an infected bird (RR 2.37, 95% CI 2.05 to 2.75). Thus in 2002, counties that reported a WNV-infected dead bird before August 4 were more than two times more likely than other counties to report a human case of WNV disease (Figure 2).

The early August date (end of the 31st week of the year) used for classifying the surveillance data was selected by empirical analysis of the WNV epidemiologic curves. This date approximates the inflection point where the rapid increase in case reports occurs. Using an earlier date for classifying the cases results in an increased value for the RR statistic but a decrease in sensitivity.

This type of analysis could possibly be refined by stratifying surveillance data by the number of birds and humans tested to compensate for variations in the intensity of the surveillance effort. Factors such as the size of the human population also may affect the number of dead birds sighted and the number of persons exposed to WNV-infected mosquitoes. Other

researchers have attempted to address these issues (6–8). In addition, we are analyzing the data to determine if the risk for human illness is greater the earlier the positive bird (or other indication of epizootic transmission) is detected.

However, the aim of this study was to see if a simple analysis of surveillance data could provide useful indicators of human disease risk. The results of our analysis suggest that, in counties where an avian epizootic is present early in the transmission season, subsequent WNV disease in humans is more likely. An early epizootic may indicate viral activity that has sufficient time to escalate to high levels before the end of the transmission season. WNV-infected dead birds found in spring or early summer thus may be a warning for increased human risk for WN viral disease.

Dr. Guptill is a senior research physical scientist at the U.S. Geological Survey. He is internationally recognized for his contributions dealing with geographic information systems research and geospatial data policy issues. Currently he is conducting spatial analysis research with colleagues from the Centers for Disease Control and Prevention to determine the environmental influences on emerging zoonotic and vector-borne diseases.

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Address for correspondence: Stephen C. Guptill, U.S. Geological Survey, 521 National Center, Reston, VA 20192, USA; fax: 703 648 4603; email: sguptill@usgs.gov

# Human Neurobrucellosis with Intracerebral Granuloma Caused by a Marine Mammal *Brucella* spp.

**Annette H. Sohn,\* Will S. Probert,†
Carol A. Glaser,\*† Nalin Gupta,\* Andrew W. Bollen,\*
Jane D. Wong,† Elizabeth M. Grace,\*
and William C. McDonald\***

We present the first report of community-acquired human infections with marine mammal–associated *Brucella* spp. and describe the identification of these strains in two patients with neurobrucellosis and intracerebral granulomas. The identification of these isolates as marine mammal strains was based on omp2a sequence and amplification of the region flanking bp26.

Brucellosis, caused by intracellular gram-negative bacteria of the genus *Brucella*, is endemic in many areas of the world. Exposure occurs through contact with infected animals, meat, or unpasteurized milk products. Neurobrucellosis is a rare, severe form of systemic infection and has a broad range of clinical syndromes (1–3). Central nervous system *Brucella* granulomas have been infrequently reported in sellar and parasellar sites and in the spinal cord (4–6).

Although a number of *Brucella* spp. cause systemic disease in humans, they have species-specific primary reservoirs. The six recognized species of *Brucella* are primarily associated with terrestrial mammals and rodents. Recently, *Brucella* has been found to cause infections in marine mammals (7,8). An expansion of the six current nomen species of *Brucella* has been proposed to include one (*B. maris*) or two (*B. pinnipediae* and *B. cetaceae*) new nomen species to categorize these strains (7,8).

To date, only one human infection with a marine mammal strain has been reported; this infection occurred in a research laboratory worker after occupational exposure (9). We present the first report of community-acquired human infection with marine mammal–associated *Brucella* spp. and describe the identification of these strains from two patients with neurobrucellosis and intracerebral granulomas.

## Case Reports

### Patient 1

Patient 1 was a previously healthy, 26-year-old Peruvian man who was evaluated in July 1985 for a 3-month history of periorbital pain, headaches, and periodic generalized tonic-clonic seizures. The initial neurologic examination was nonfocal, but subsequent computerized tomography scan showed a 5x5-cm enhancing mass in the left frontoparietal region associated with midline shift.

At the time of surgical biopsy, frozen section histology raised the possibility of a high-grade astrocytoma or lymphoma, prompting resection of a 3x3-cm well-circumscribed left frontal lobe mass. Final examination of pathologic specimens showed granulomas with multinucleated giant cells (Figure 1). Bacterial, fungal, and acid-fast bacilli stains were negative. Based on these pathologic findings and concern that the patient may have had tuberculosis, treatment with isoniazid, rifampin, and ethambutol was begun. Serologic tests for *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Paracoccidioides brasiliensis* were negative. *Toxoplasma gondii* serologic titers were weakly positive at 1:64.

On postoperative day 39, fungal tissue cultures became positive for a *Brucella* spp., preliminarily identified as *B. melitensis*. The patient's antimicrobial treatment was changed to tetracycline and rifampin and was continued for 2 months. An initial serologic titer for *Brucella* was positive at 1:160 by tube agglutination assay. A follow-up serologic titer obtained in January 1986 was negative (<1:20).
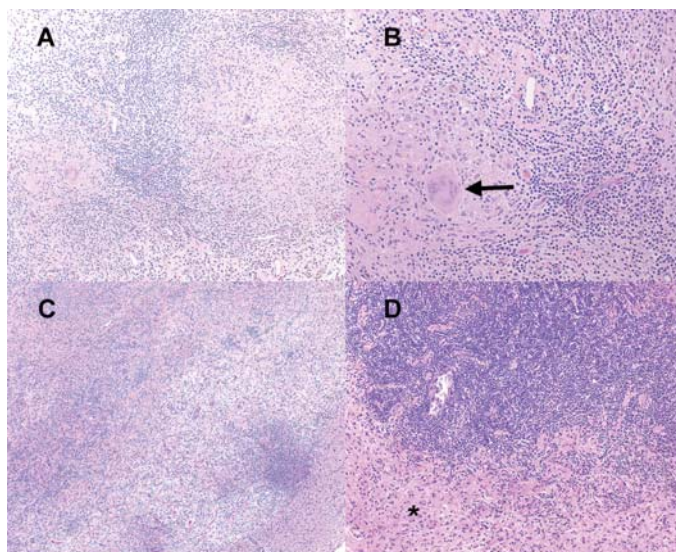


Figure 1. Hematoxylin and eosin–stained sections from patient 1 (panels A, B) and patient 2 (panels C, D). Note the predominantly lymphohistocytic infiltrate forming large granulomas (A, original magnification 100x); well-formed giant cells (B, arrow, original magnification 200x); lymphohistiocytic infiltrates distorting brain parenchyma and forming vague granulomas (C, original magnification 40x); and the dense astrogliosis at the interface between granulomatous inflammation and surrounding brain parenchyma (D, asterisk, original magnification 100x).

\*University of California, San Francisco, San Francisco, California, USA; and †California Department of Health Services, Richmond, California, USA

Three months before his initial evaluation, the patient had immigrated to the United States from Lima, Peru. His diet included regular consumption of unpasteurized cow or goat cheese (*queso fresco*) and occasional consumption of raw shellfish (*ceviche*). He denied eating other raw or significantly undercooked meat. He had frequently swum in the Pacific Ocean from December through March but recalled no direct contact with marine mammals.

## Patient 2

Patient 2 was a 15-year-old Peruvian boy seen in September 2001 with a 1-year history of headaches, nausea, vomiting, and progressive deterioration in visual function. A magnetic resonance imaging (MRI) scan performed in April 2001 showed several large, irregular enhancing mass lesions involving the left occipital and parietal lobes (Figure 2). He had come to the United States in September for further evaluation. On neurologic examination, the patient had a right homonymous hemianopsia, optic nerve atrophy, and major visual impairment (left eye—20/100; right eye—20/200). Repeat MRI showed several irregular areas of enhancement in the left parietooccipital area associated with marked brain edema, left-to-right shift, and mass effect (Figure 2).

The patient was taken to surgery, where a firm avascular mass was found beneath a 0.5-cm layer of softer gliotic cortical tissue. Frozen sections showed lymphohistiocytic infiltrates with granuloma formation. Specimens for cultures and histopathologic examination were obtained. Bacterial, fungal, and acid-fast bacilli stains were negative. Final histopathologic examination showed numerous granulomas with multinucleated giant cells but no organisms (Figure 1).

Serologic test results for *Brucella*, *T. gondii*, and *Taenia soleum* were negative. On postoperative day 8, mycobacteria cultures (BacT/ALERT MP; Organon Teknika Corp., Durham, NC) showed growth of a gram-negative coccobaccillus, later confirmed as *Brucella* spp. Treatment with rifampin, doxycycline, and intravenous gentamicin was begun. After 1 week, the gentamicin was discontinued per current recommendations, and trimethoprim-sulfamethoxazole was started. Follow-up imaging 7 months later demonstrated resolution of the enhancing areas and edema, with residual areas of brain atrophy (Figure 2). The patient's vision improved, but some visual acuity deficits persisted. Anti-brucella therapy continued for 1 year.

The patient lived in a small town 7 hours from Lima and had not traveled outside of the country before coming to the United States. His diet included regular consumption of *queso fresco* and occasional *ceviche* but no other raw meats. He reported no direct contact with marine mammals and seldom visited the Peruvian coast.

## Materials and Methods

Bacterial isolates were identified as *Brucella* spp. by using a real-time, 5′ exonuclease (TaqMan) assay, based on a well-characterized polymerase chain reaction (PCR) assay that targets a highly-conserved 223-bp region of a gene (*bcsp31*)
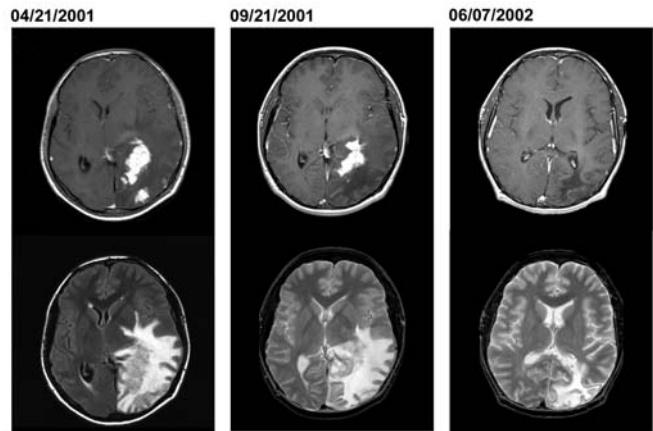


Figure 2. Axial MRI (magnetic resonance imaging) from patient 2 obtained when first seen in Peru (April 21, 2001), before surgical biopsy in the United States (September 21, 2001), and 7 months after start of treatment (June 7, 2002). The top images are postgadolinium-enhanced, T1-weighted images, which demonstrate resolution of one of the irregular areas of enhancement over time. The bottom images are T2-weighted images (image from April 21 is from a fluid attenuated inversion recovery [FLAIR] sequence), which demonstrate the extensive brain edema associated with these lesions. The right bottom image shows resolution of the brain edema but persistent brain atrophy.

encoding a 31-kDa immunogenic *B. abortus* protein (10). Amplified product was detected through the design of a dual-labeled hybridization probe, 5′-CCGGTGCCGTTATAGGCC-CAATAGG (5′, 6-carboxyfluorescein label; 3′, 6- carboxyte-tramethylrhodamine label). Real-time detection of amplified product was performed with a LightCycler (Roche Applied Science, Indianapolis, IN) by using the following amplification parameters: 2 min at 50°C, 10 min at 95°C, and 30 cycles of 95°C for 10 s and 60°C for 30 s. Fluorescence was monitored at 530 nm.

After identifying a bacterial isolate as *Brucella* spp., we attempted further classification to the species level by using real-time PCR assays to detect *B. abortus*, *B. melitensis*, and biovar 1 of *B. suis* (11). We used a PCR assay targeting the *bp26* gene, performed as described by Cloeckaert et al. (12), to discriminate between terrestrial strains and marine mammal strains of *Brucella*.

Partial DNA sequencing of *omp2a* was also used for *Brucella* isolate classification (13). Amplification and sequencing of a 519-bp region of the *omp2a* gene were performed by using the primers 5′-GGGTGGCGAAGACGTTGACAA and 5′-AACCGTTGGCGCCCTATGC. Dye terminator cycle sequencing (Applied Biosystems, Foster City, CA) was performed according to the manufacturer's recommendation; the sequencing reactions were analyzed on an ABI 377 DNA sequencer.

## Results

### Histopathology

Both cases demonstrated well-developed, granulomatous inflammation with accompanying astrogliosis. Patient 1

showed dense perivascular lymphoplasmacytic infiltrates with nonnecrotizing granulomas containing epithelioid and spindle-shaped macrophages and occasional well-formed giant cells (Figure 1). Tissue Gram, Fite, and silver methenamine stains showed no definite organisms. Patient 2 showed florid granulomatous inflammation with large areas of epithelioid and spindle-shaped histiocytes containing foci of necrosis surrounded by dense chronic inflammation (Figure 1). Giemsa, Steiner, Warthin-Starry, tissue Gram, Grocott methenamine silver, and Fite stains did not detect organisms. Giant cells were not observed in patient 2. Masson trichrome stain showed accompanying marked fibrosis.

## Molecular Microbiology

Presumptive *Brucella* isolate 01A09163 was isolated from the brain biopsy of patient 2 and was submitted to the Microbial Diseases Laboratory (the microbiology reference laboratory for the State of California) for laboratory confirmation. Identification of *Brucella* spp. was confirmed by a 5′-exonuclease assay that targets a 223-bp region of the *bcsp31* gene. Isolates confirmed as *Brucella* were then further classified as *B. abortus*, *B. melitensis*, or *B. suis* biovar 1 by the real-time PCR assays described by Redkar et al. (11). Strain 01A09163 tested negative by these assays, indicating that this isolate likely represented a different species of *Brucella*.

A review of Microbial Diseases Laboratory records showed a second *Brucella* strain, 85A05748, associated with an intracerebral granuloma. This strain was isolated in 1985 from the brain lesion of patient 1. Given the clinical similarities in these two cases, strain 85A05748 was tested by PCR. Like strain 01A09163, strain 85A05748 was negative in the *B. abortus*, *B. melitensis*, and *B. suis* biovar 1 assays.

To aid in the identification of these strains to the species level, a portion of the *omp2a* locus was amplified and sequenced. Between the two strains, the *omp2a* sequences were identical and indicated that the two strains were likely of the same species. A BLAST search of the National Center for Biotechnology Information databases established that the *omp2a* sequence of these two strains was identical to the *omp2a* sequence derived from *Brucella* sp. B2/94 (GenBank accession no. AF300819), a marine mammal strain of *Brucella* isolated from a common seal (7). Compared to other *Brucella* strains, one or more polymorphisms were noted in the *omp2a* sequence of 01A09163 and 85A05748. This observation suggested that strains 01A09163 and 85A05748 were most closely related to a marine mammal strain, rather than a terrestrial strain, of *Brucella*.

To verify that strains 01A09163 and 85A05748 were genetically related to strains of *Brucella* derived from marine mammals, we performed a PCR assay targeting the *bp26* gene (12). Marine mammal strains of *Brucella* possess an IS711 element immediately downstream of *bp26*, whereas the sequence of terrestrial strains does not. Consequently, amplification of the region surrounding *bp26* yields a much larger DNA fragment for marine mammal strains as compared to the amplification

product produced by terrestrial strains of *Brucella*. As shown in Figure 3, amplification of the *bp26* gene from strains 01A09163 and 85A05748 yielded a 1,900-bp product that was diagnostic for marine mammal strains of *Brucella*. Amplification of *bp26* from a terrestrial strain, *B. abortus*, produced the expected 1,024-bp product.

## Discussion

Our report of serious central nervous system disease caused by a marine mammal *Brucella* strain confirms that these organisms can cross over from their primary hosts to humans in a community setting. *Brucella* infections have been documented in studies of marine mammals that died after being stranded ashore (7,8). Although a formal nomenclature has not yet been established, evidence is sufficient to support the expansion of the *Brucella* genus. Studies of DNA polymorphisms at the *omp2* locus indicate that more than one species may be represented in this group (8). When the proposed classification scheme of Cloeckaert et al. (8) is used, partial *omp2a* sequence suggests that the two isolates from our study are most closely related to *B. pinnipediae*, a seal strain of *Brucella*.

Despite a more than 15-year separation, these cases have a number of epidemiologic, clinical, and histopathologic similarities. Both patients had recently immigrated from Peru. They denied significant exposure to marine mammals. Neuroimaging and pathology studies were very similar. Marked granulomatous inflammation was observed in each biopsy, but histopathologic studies did not reveal the organism in tissue sections. Definitive diagnosis was subsequently made by bacterial isolation.

Notably, *Brucella* spp. antibody was not elevated in patient 2. Despite his prolonged duration of illness and extensive central nervous system involvement, the patient's steroid medications, unknown host factors, or low immunogenicity of marine mammal strains may be responsible for his lack of serologic response, emphasizing the importance of direct isolation.

Neurobrucellosis develops in <5% of patients with *Brucella* infection (1). The most frequent clinical syndromes associated with acute infection are meningitis or meningoencephalitis (1–3). Mass lesions within the brain parenchyma are extremely uncommon but have been documented radiographically (6) and
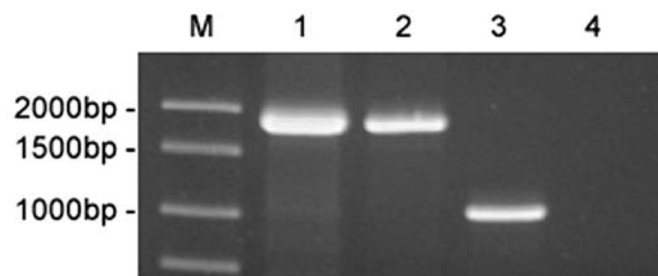
Figure 3. Amplification of bp26 from marine mammal and terrestrial strains of *Brucella*. The amplification products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. Lane 1, strain 01A09163; lane 2, strain 85A05748; lane 3, *B. abortus* ATCC 23448; lane 4, no template control. DNA ladder is shown in Lane M.

pathologically (14). Patient 1 underwent granuloma resection before medical therapy and did not have relapse of infection. Patient 2 was treated with medical therapy. Such infections, despite widespread central nervous system extension, could possibly be treated medically, reducing potential long-term neurologic complications of resection. Furthermore, the indolent nature of these infections suggests that early detection and treatment could prevent the long-term consequences of a chronic intracranial inflammatory process.

These cases raise important issues about the epidemiology of human *Brucella* infection related to transmission of nonterrestrial strains. In the absence of a direct association with marine mammals, more traditional zoonotic sources of infection may be involved. Experimental infection of dairy cattle with a marine mammal strain of *Brucella* has been documented (15). Given the extensive Peruvian coastline, marine mammal strains of *Brucella* could conceivably have been transmitted to domestic animals and wildlife that reside nearby.

What degree of pathogenicity these strains may have in human infection is unclear. Further study of infection in both humans and marine mammals is needed to characterize the spectrum of disease and the potential for communicability. Such studies may be facilitated by the implementation of new laboratory tests to rapidly identify marine mammal strains of *Brucella*.

## Acknowledgments

Dr. Sohn is a fellow in the Division of Pediatric Infectious Diseases at the University of California, San Francisco. Her research interests include the clinical and molecular epidemiology of hospital-acquired infections in high-risk pediatric populations and antimicrobial utilization and resistance.

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Address for correspondence: Annette H. Sohn, 500 Parnassus Avenue, MU 407E, San Francisco, California 94143-0136, USA; fax: (415) 476-1343; email: asohn@itsa.ucsf.edu

# Antimicrobial Growth Promoters and *Salmonella* spp., *Campylobacter* spp. in Poultry and Swine, Denmark

**Mary C. Evans\* and Henrik C. Wegener\***

The use of antimicrobial growth promoters in Danish food animal production was discontinued in 1998. Contrary to concerns that pathogen load would increase; we found a significant decrease in *Salmonella* in broilers, swine, pork, and chicken meat and no change in the prevalence of *Campylobacter* in broilers.

Antimicrobial growth promoters are antimicrobial drugs added to animal feed to enhance growth and improve feed efficiency of food animals. In the United States, the use of antimicrobial growth promoters also includes elements of prophylaxis, which are not allowed in Europe. Antimicrobial growth promoters have been widely used in Danish food animal production since the 1970s. On February 15, 1998, the Danish cattle and broiler industries, reacting to consumer concerns over food safety, voluntarily stopped the use of all antimicrobial growth promoters. The pig industry stopped using the growth promoters in pigs over 35 kg; all use was phased out in 1999.

Despite concerns that no longer using the growth promoters would have a wide range of negative effects (e.g., an increase in disease and death, poor growth rates, increased feed consumption, increased fecal shedding, and enhanced shedding or carriage of foodborne pathogens such as *Salmonella* or *Campylobacter*), producers in the broiler and pig industries discontinued the use. Studies have shown that antimicrobial drugs reduce part of the intestinal flora while potentially decreasing pathogen shedding (1–3). For these reasons, producers believed that removing antimicrobial growth promoters could cause human pathogenic intestinal bacteria in food animals to increase. Another concern was that increased fecal shedding could lead to contamination of carcasses at slaughter and increased risk for foodborne infection in humans.

Our study examines the effect of discontinued use of antimicrobial growth promoters on the prevalence of *Salmonella* in Danish broiler flocks, chickens after slaughter, swine herds, and pork end products. We also examine the effect on *Campylobacter* in Danish broiler flocks.

## The Study

Data for this analysis were obtained from routine monitoring programs in Danish broilers and swine. Approximately 450,000 broiler and chicken samples and 830,000 swine and pork samples are tested each year. A detailed description of sample collection methods and numbers of samples is available (4).

Broiler flocks have been tested for *Salmonella* since 1989 and for *Campylobacter* since the end of 1995. Initially, flocks were tested for *Salmonella* by the collection of 16 fecal samples; however, since June 2000, five "sock samples" (Samples were taken by placing a sock over the collector's shoes. The collector walks through the poultry house; the socks absorb fecal samples) have been collected. Antemortem samples are obtained 2–3 weeks before slaughter by collecting five pairs of sock samples per flock (4). Before November 2000, postmortem *Salmonella* sampling was conducted by examination of five pooled swab samples each consisting of 10 neck-skin samples per flock. After November 2000, postmortem sampling came from batches of poultry parts. Because of this change, we excluded postmortem data (after November 2000) from analysis. Broiler flocks are monitored for *Campylobacter* by examination of cloacal swabs from 10 birds per flock or batch at slaughter.

Since June 1995, swine herds have been continuously monitored for *Salmonella* by serologic testing of "meat juice" (10g of muscle tissue are collected from the neck, diaphragm or tenderloin of the animal. This sample is placed into a container consisting of an upper coffee-filter like part to hold the meat and a lower tube-like part. The container is frozen overnight at –20°C and subsequently allowed to thaw at 4°C for 24 hours, causing release of the meat juice into the lower part of the tube. This juice is then tested for *Salmonella* antibodies) samples from each herd producing >100 finishers (pigs 30–50 kg) per year. In July 2001, this requirement changed to herds producing >200 finishers per year. The number of samples taken is dependent on herd size. Based on serologic results, herds are placed into categories. Level 1 herds have no or few seroreactors (animals that test positive for *Salmonella*), and no intervention is required. Level 2 herds have a higher proportion of seroreactors, and the herd owner must seek advice on reducing the prevalence of *Salmonella*. Level 3 herds have a large proportion of seroreactors, and the herd owner must seek advice and slaughter under special hygienic conditions.

Since July 1993, *Salmonella* in pork has been measured by monthly samples of cuts of meat from slaughterhouses. The number of samples depends on the number of animals slaughtered. In January 2001, the sampling method changed to carcass swabs. Consequently, data from 2001 were not included in this analysis (4).

Data were divided into two periods: before (P1) and after (P2) withdrawal of antimicrobial growth promoters. To account for factors such as seasonality, equal time periods were used for comparison; however, periods varied from 12 to 36 months, depending on availability of data. Although the voluntary discontinuation on purchasing antimicrobial growth promoter-

\*Danish Veterinary Institute, Copenhagen, Denmark

containing feed for broilers was initiated on February 15, depletion of feed stocks may have taken several weeks. To account for this and for the uneven withdrawal of antimicrobial growth promoters in pigs, data from 1998 (broilers) and 1998–1999 (swine) were excluded from analysis. The resulting time periods are found in Table 1.

For this analysis, the mean prevalence of positive flocks (broilers), the percentage of herds classified as level 2 or level 3 (swine herds), and the percentage of positive samples (pork) were obtained by month for P1 and P2. Using these data, the mean prevalence for P1 and P2 were calculated and differences in means were evaluated by a t test by using SAS version 8.0 (SAS Institute, Inc., Cary, NC). The prevalence of *Salmonella* and *Campylobacter* in broilers and swine between 1995 and 2001 is shown in the Figure.

The mean percentage of broiler flocks testing positive for *Salmonella* during P1 (before withdrawal) was 14.4% (range 3.7% to 33.6%) for antemortem examination and 17% (range 8.1% to 38.8%) for postmortem (Table 2) P2 samples averaged 2.4% (range 0.2% to 5.8%) and 4.9% (range 0.7% to 24.9%), respectively. A comparison of means showed that the prevalence of *Salmonella* was significantly lower in the period following withdrawal of antimicrobial growth promoters for both antemortem (p<0.0001) and postmortem samples (p<0.0001). The average percentage of broiler flocks testing positive for *Campylobacter* during P1 was 35.3% (11.4% to 64.%8) and P2 was 40.8 (18% to 77%). No statistical difference existed in the mean prevalence between the two periods (p=0.2470).

The percentage of swine herds classified as level 2 or level 3 during P1 was 5% (4.2% to 6.2%) and 3.3% (2.5% to 4.4%) for P2. A comparison of means showed that the average percentage of swine herds classified as level 2 or 3 was significantly lower in the period following the withdrawal of antimicrobial growth promoters (p<0.0001). The percentage of *Salmonella* isolated from fresh pork samples dropped from 1.1% (0.5% to 1.8%) in P1 to 0.8% (0.4% to 1.5%) in P2. Although the change is small, the period following the withdrawal of antimicrobial growth promoters was significantly lower (p=0.0290).

## Conclusions

Contrary to concerns that withdrawal of antimicrobial growth promoters would cause an increase in pathogen load, we found a decrease in *Salmonella* prevalence in broilers, chicken, swine, and pork and no change in the prevalence of *Campylobacter* in broilers. Previous studies on this topic have shown mixed results. Two observational studies found that
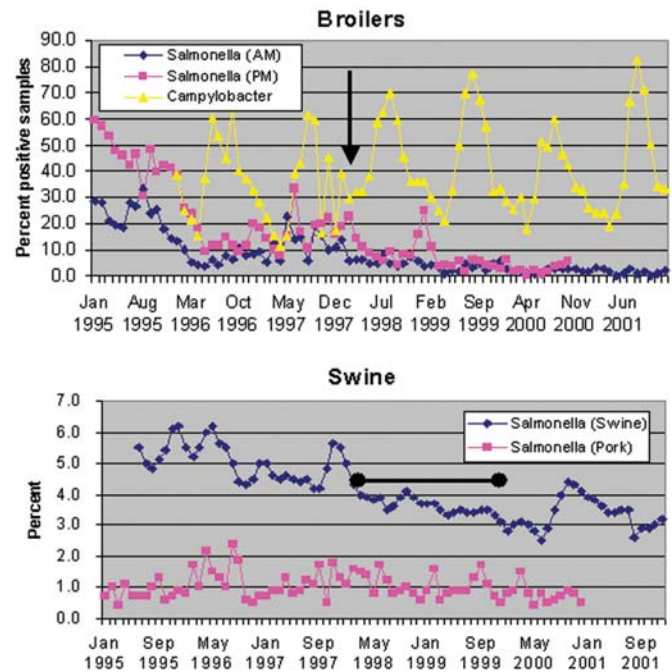


Figure. Prevalence of *Salmonella* and *Campylobacter* in Danish broiler flocks, chicken meat, swine herds, and pork products, 1995-2001. The arrow indicates February 15, 1998 the date of the voluntary stop of AGP use in broilers. The bar indicates the time period during which antimicrobial growth promoters were withdrawn from use in swine herds.

penicillin given to swine increased total bacterial and *Enterobacteriaceae* counts (5,6). Other experiments found that avoparcin increased *Salmonella* shedding in broilers and excretion rates had a dose-response effect with increasing concentrations of avoparcin (7,8). A series of experiments in broilers showed that avoparcin, nitrovin, tylosin, flavomycin, and lincomycin caused increased shedding of *Salmonella* in most experiments, while virginiamycin and bacitracin had little or no effect and sodium arsenilate decreased shedding (9–11). Holmberg et al. found that both avoparcin and monensin reduced shedding of *S.* Infantis in broilers but a combination of the two increased shedding (12). Bolder et al. showed that flavophospholipol and salinomycin decreased *Salmonella* shedding in broilers but had no significant effect on the shedding of *C. jejuni* (13).

Our study is unique because we included a large sample of animals under natural conditions, included both animals and products, and examined the combined effect of many antimicrobial growth promoters. However, several factors should be kept in mind when interpreting the results. First, our analysis

| Table 1. Description of time intervals used for data analysis, by species and pathogen[a] | | | |
|---|---|---|---|
| Species | Pathogen | Period 1 (P1) | Period 2 (P2) |
| Broilers | *Salmonella* (antemortem ) | Jan 1995–Dec 1997 | Jan 1999–Dec 2001 |
| | *Salmonella* (postmortem) | Jan 1996–Oct 1997 | Jan 1999–Oct 2000 |
| | *Campylobacter* | Jan 1996–Dec 1997 | Jan 1999–Dec 2000 |
| Swine | *Salmonella* | Jan 1996–Dec 1997 | Jan 2000–Dec 2001 |
| | *Salmonella* (pork) | Jan 1997–Dec 1997 | Jan 2000–Dec 2000 |

[a]Period 1 and 2 refer to the time periods before (P1) and after (P2) the withdrawal of antimicrobial growth promoters

Table 2. Comparison of the average prevalence of *Salmonella* and *Campylobacter* in broiler flocks, chicken meat, swine herds, and pork products before and after withdrawal of antimicrobials for use as growth promoters

|  | Period 1[a] mean (range) | Period 1 standard deviation | Period 2[b] mean (range) | Period 2 standard deviation | p value |
|---|---|---|---|---|---|
| *Salmonella* |  |  |  |  |  |
| Broilers (antemortem) | 14.4 (3.7–33.6) | 8.3902 | 2.4 (0.2–5.8) | 1.3215 | <0.0001 |
| Broilers (postmortem) | 17.0 (8.1–38.8) | 7.9125 | 4.9 (0.7–24.9) | 5.0493 | <0.0001 |
| Swine | 5.0 (4.2–6.2) | 0.5904 | 3.3 (2.5–4.4) | 0.5347 | <0.0001 |
| Pork | 1.1 (0.5–1.8) | 0.3895 | 0.8 (0.4–1.5) | 0.2839 | 0.0290 |
| *Campylobacter* |  |  |  |  |  |
| Broilers (antemortem) | 35.3 (11.4–64.8) | 16.6290 | 40.8 (18.0–77.0) | 16.1185 | 0.2470 |

[a]Before withdrawal of antimicrobial growth promoters.
[b]After withdrawal of antimicrobial growth promoters.

cannot elucidate the impact that withdrawal of an individual antimicrobial growth promoter had on a particular pathogen or in a particular species. In addition, since avoparcin was withdrawn in 1995, any immediate effects seen from its discontinued use will be demonstrated in P1 of our study instead of P2. Despite a change in sampling methods for broilers in June 2000 and swine herds in July 2001, these data were included in analysis. Both changes increased the sensitivity of sampling, in theory leading to a higher prevalence. Since this change occurred during P2, it would tend to bias our results toward the null; thus, including these samples gives our study a more conservative result. Finally, our study only describes the prevalence of *Salmonella* and *Campylobacter* after the withdrawal of antimicrobial growth promoters. Effects such as productivity, changes in therapeutic antimicrobial drug use and economic impact are described in another study (14).

Our findings only show a temporal relationship between withdrawal and reduction, and one should be cautious not to infer causality. The fact that the decrease was seen before and during the use of antimicrobial growth promoters suggests that other factors play a role. The most obvious of these factors is the effect of the ongoing surveillance and control programs in food-producing animals. Programs in broilers and swine, described each year in the Annual Report on Zoonosis (4), have been in effect since the late 1980s and mid-1990s and have made a substantial impact on reducing the prevalence of *Salmonella* in primary food production. What is clearly shown from this analysis is that *Salmonella* and *Campylobacter* rates have not increased in food animal carriers since antimicrobial growth promoters were withdrawn in 1998. This finding, combined with evidence that the withdrawal has taken place without remarkably noticeable effects on the productivity in broilers (15) and swine, is of particular importance in light of the emerging problem of antimicrobial drug-resistant human pathogenic organisms, which are associated with the use of antimicrobial growth promoters.

Ms. Evans holds a master's degree in public health epidemiology from Emory University. She was formerly employed in the foodborne diseases branch at Centers for Disease Control and Prevention and currently works as a research epidemiologist at the Danish Zoonosis Centre in Copenhagen, Denmark. Her research interests include infectious disease epidemiology and international health issues.

Dr. Wegener is a professor of zoonoses epidemiology at the Danish Veterinary Institute and Royal Veterinary and Agricultural University of Copenhagen, Denmark. His research interests involve epidemiology of foodborne zoonoses, antimicrobial resistance in the food chain, and microbial risk assessment.

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Address for correspondence: Mary Christine Evans, Danish Zoonosis Centre, Danish Veterinary Institute, Bülowsvej 27, DK-1790 Copenhagen V, Denmark; fax: +(45) 35 30 03 77; email: mce@vetinst.dk

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Seasonal Cycles and Susceptibility to Infections

# First Incursion of *Salmonella enterica* Serotype Typhimurium DT160 into New Zealand

Craig N. Thornley,* Greg C. Simmons,†
Megan L. Callaghan,† Carolyn M. Nicol,*
Michael G. Baker,* Kylie S. Gilmore,*
and Nicholas K.G. Garrett‡

An outbreak of human *Salmonella enterica* serotype Typhimurium DT160 infection in New Zealand was investigated from May to August 2001. Handling of dead wild birds, contact with persons with diarrheal illness, and consumption of fast food were associated with infection. Contaminated roof-collected rainwater was also detected.

Although rates of reported salmonellosis in New Zealand are relatively high for an industrialized country (average of 46.1 cases per 100,000 population per year, 1995–2001 [1]), strict biosecurity has prevented extension to New Zealand of the global pandemics of *Salmonella enterica* serotype Enteritidis and *S. enterica* serotype Typhimurium definitive type (DT) 104 (2). *S.* Typhimurium DT160 was first identified as a human pathogen in New Zealand in 1998 (3). Since July 2000, the incidence of human infection with this serotype has increased markedly, and the geographic distribution of cases has progressively expanded from New Zealand's South to North Islands (Baker et al., unpub. data). However, routes of disease transmission have not been identified. The epidemic occurred in parallel with an epizootic leading to deaths in wild birds, mainly sparrows, due to septicemia caused by *S.* Typhimurium DT160 (4). We report an investigation of an outbreak of *S.* Typhimurium DT160 infection in humans.

## The Study

In May 2001, 24 cases of *S.* Typhimurium DT160 salmonellosis were reported in the Auckland region compared with an average of four sporadic cases each month with this serotype in the previous 7 months. Raw and undercooked egg consumption was commonly reported by the first 10 case-patients interviewed. A case-control study and environmental investigation were undertaken to identify the vehicle of infection and source of the outbreak. Recognizing the potential for a widely dispersed foodborne outbreak, we expanded the investigation throughout New Zealand.

*Institute of Environmental Science & Research Limited, Kenepuru Science Centre, Porirua, New Zealand; †Auckland Regional Public Health Service, Auckland District Health Board, Auckland, New Zealand; and ‡Mt. Albert Science Centre, Auckland, New Zealand

Cases were identified from disease reports and isolates received by the national reference laboratory. We defined a case as diarrhea (≥3 loose stools in a 24-hour period) or vomiting after April 28, 2001, with a stool specimen positive for *S.* Typhimurium DT160. Patients were excluded if they had a history of contact with another person with culture-confirmed *S.* Typhimurium DT160 infection, or if they had a history of recent overseas travel. Each case was matched with two controls found from randomly drawn telephone numbers, matching for neighborhood and age (<1, 1–4, 5–14, >14 years).

Patients and controls were interviewed by telephone. The questionnaire covered symptoms (patients only) and contact with other symptomatic persons, bird or animal contact, and food consumption in the 3-day period before onset of illness (cases and controls). Parents or guardians were interviewed on behalf of children ages ≤12 years. A matched univariate analysis was performed with SAS software (5). Stepwise conditional logistic regression analyses were performed, also using SAS, to identify the combination of variables that best explained the differences between case-participants and controls.

Samples from the drinking water supply of case-patients with a history of recent consumption of nonreticulated water were collected and tested for coliforms and *S. enterica* by using standard methods (6). Brands of eggs eaten raw within the incubation period were sampled at random from retail displays at the case-patients' purchase site. At least six shell eggs were collected in each sample. Eggshell surfaces and contents were tested with standard methods (7). Broken or cracked eggs were excluded from analysis. *Salmonella* isolates were serotyped by using the Kauffman-White scheme (8) and *S.* Typhimurium isolates were phage typed by using the Laboratory of Enteric Pathogens method (9).

From May to August 2001, a total of 170 case-patients meeting the case definition were identified. Of these, 119 (70%) agreed to participate and were enrolled in the study, along with 235 matched controls. The median age of case-patients was 8 years (range 4 months to 90 years), and 57% were female. The most frequently reported symptoms were diarrhea (97%), abdominal pain (77%), excessive tiredness (67%), and fever (66%). Vomiting in the absence of diarrhea was reported by one (0.8%) patient. The median duration of illness was 7 days (range 1–44 days); 17 (15%) patients were hospitalized, and none died. Case-patients and controls did not differ significantly according to age, sex, immunosuppressive therapy, treatment to reduce gastric acidity, or use of antibiotics. All *S.* Typhimurium DT160 isolates were sensitive to ampicillin, cephalothin, chloramphenicol, ciprofloxacin, co-trimoxazole, gentamicin, streptomycin, sulfonamides, tetracycline, and trimethoprim.

Seven exposures had significant univariate associations with increased risk for illness (Table). Four represented different levels of contact with other persons with gastrointestinal illness (i.e., within 28 days of illness onset; within 3 days of onset; within the household; or outside the household). Direct handling of dead wild birds, consumption of fast food, and con-

Table. Frequency of selected exposures among case-patients and controls, *Salmonella* Typhimurium DT160 outbreak, New Zealand, 2001

| Exposure | No. (%) | | Matched OR | 95% CI | p value |
|---|---|---|---|---|---|
| | Case-patients n = 119 | Control n = 235 | | | |
| Direct handling of dead wild birds | 13 (10.9) | 3 (1.3) | 12.28 | 2.76 to 54.63 | 0.001 |
| Exposure to person with D&V in household in 3 d before illness in case-patient | 7 (5.9) | 3 (1.3) | 4.67 | 1.21 to 18.05 | 0.03 |
| Exposure to person with D&V in any setting in 3 d before illness in case-patient | 14 (11.8) | 8 (3.4) | 3.81 | 1.53 to 9.49 | 0.004 |
| Exposure to person with D&V in household in 28 d before illness in case-patient | 11 (9.2) | 8 (3.4) | 3.11 | 1.13 to 8.54 | 0.03 |
| Exposure to person with D&V in any setting in 28 d before illness in case-patient | 28 (23.5) | 20 (8.5) | 3.05 | 1.64 to 5.69 | <0.001 |
| Consumption of food at a large gathering | 24 (20.2) | 23 (9.8) | 2.44 | 1.27 to 4.68 | 0.007 |
| Consumption of any fast food | 69 (58.0) | 111 (47.2) | 1.69 | 1.04 to 2.75 | 0.04 |
| Drinking of roof-collected rainwater | 12 (10.1) | 19 (8.1) | 2.35 | 0.55 to 10.05 | 0.25 |
| Consumption of raw eggs | 5 (4.2) | 3 (1.3) | 3.33 | 0.80 to 13.95 | 0.10 |

[a]DT, definitive type; D&V, diarrhea and vomiting; OR, odds ratio; CI, confidence interval.

sumption of food at a large gathering, such as at a party or large barbecue, were also significantly associated with illness. Six of those who had handled dead birds were <5 years of age. Others who had handled dead birds had no characteristics in common. After stepwise regression, contact with a person with gastrointestinal illness in the 28 days before onset of illness in the case-patient (adjusted odds ratio [OR] 2.8; 95% confidence interval [CI] 1.4 to 5.4), handling of dead wild birds (adjusted OR 10.5; 95% CI 2.3 to 47.5), and consumption of fast food (adjusted OR 1.7; 95% CI 1.0 to 2.9) had independent significant associations with illness.

Twelve case-patients throughout New Zealand indicated that they had drunk water from nonreticulated and untreated water sources. Eight sources were sampled in Auckland. Seven patients used roof-collected rainwater, and one used rainwater plus water from a dam. Four sampled sources, used by five patients, contained *S.* Typhimurium DT160. All were from roof-collected rainwater. Four of the five patients who had eaten raw eggs could identify the retail brand and outlet of purchase. These four patients had purchased six different brands of eggs from seven different retail outlets. Samples for two brands were positive for *S.* Thompson, both from shell surface washings.

## Conclusions

Epidemiologic investigation of an outbreak of *S.* Typhimurium DT160 infection in New Zealand from May to August 2001 found that contact with dead wild birds, contact with other persons with gastrointestinal illness, and consumption of fast food were all significantly associated with illness. In addition, *S.* Typhimurium DT160 was found in roof-collected rainwater drunk by five patients.

*S.* Typhimurium DT160 had been previously identified as the cause of large numbers of sparrow deaths in New Zealand in 2000, and analysis by pulsed-field gel electrophoresis (using the method described by Barrett et al. [10] and restriction enzyme *Xba*I) demonstrated that bird and human isolates in 2000 were indistinguishable (4). In our study, information was not collected on exposure to environments contaminated by wild bird feces, such as parks and play areas, a fact that may have underestimated the avian contribution to human illness. *S.* Typhimurium DT160 has previously been recognized as a bird pathogen in Canada (11) and in England (12). Before its emergence in New Zealand, the human *S.* Typhimurium DT160 infection had only been reported in the context of a 1979 institutional outbreak in the United Kingdom, linked to food contamination by sparrow droppings (13).

Consumption of undisinfected water has previously been identified as a risk factor for salmonellosis linked to bird transmission (14). This risk factor was not confirmed by our case-control study, despite the finding of *S.* Typhimurium DT160 in roof-collected rainwater. This discrepancy is probably because case-patients and controls were matched by neighborhood, and types of water sources are usually consistent within neighborhoods.

The association of illness with contact with another person with gastrointestinal illness is likely underestimated because secondary salmonellosis cases were excluded. Consumption of fast food was associated with illness; however, no single type of food outlet or food was identified. Case-patients were equally likely to have eaten food from chain fast-food restaurants as from family-owned fast-food outlets. Consumption of fast food may have occurred in environments contaminated by bird feces, or the foods themselves may have been contaminated, either during production or by infected foodhandlers (15).

Sampling and recall bias may have influenced the results of this study. Asymptomatic *Salmonella* carriers would not have been excluded from selection as controls, potentially reducing the magnitude of observed associations. Recall may have been influenced by delays between exposure and interview, although participants were asked to refer to a memory aid (personal diary or calendar). Recall of unusual exposures is less likely to have been affected.

The investigation successfully excluded a single common source exposure for this outbreak and instead suggested that multiple exposures contribute to *S.* Typhimurium DT160 infections in New Zealand. Strategies for addressing these exposures include routine treatment of roof-collected rainwater, hygienic

disposal of dead birds, and promotion of hand-hygiene protocols and sick foodhandler policies in fast-food outlets. The source of this incursion of *S.* Typhimurium DT160 into New Zealand remains unknown: Bird isolates have been exclusively from nonmigratory birds, *S.* Typhimurium DT160 has not been identified in neighboring countries in the Pacific Basin, and early case-patients did not have a history of overseas travel.

### Acknowledgments

Dr. Thornley is a public health medicine specialist in the Auckland Regional Public Health Service, Auckland, New Zealand. He works in tuberculosis control and has additional research interests in zoonoses, outbreak detection, and investigation methods.

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Address for correspondence: Craig N. Thornley, Auckland Regional Public Health Service, Private Bag 92605, Symonds Street, Auckland, New Zealand; fax: +64 9 630 7431; email: craigt@adhb.govt.nz

# Automated Ribotyping and Pulsed-Field Gel Electrophoresis for Rapid Identification of Multidrug-Resistant *Salmonella* Serotype Newport

**John Fontana,\* Alison Stout,\* Barbara Bolstorff,\* and Ralph Timperi,\***

In a series of 116 *Salmonella enterica* Newport isolates that included 64 multidrug-resistant (MDR) isolates, automated ribotyping and pulsed-field gel electrophoresis (PFGE) discriminated MDR *S.* Newport with a sensitivity of 100% and 98% and specificity of 76% and 89%, respectively. Clustering of PFGE patterns (but not ribotyping) linked human and bovine cases. Automated ribotyping rapidly identified the MDR strain, and PFGE detected associations that aided epidemiologic investigations.

An eight-drug resistant strain of *Salmonella enterica* serotype Newport (multidrug-resistant [MDR] *S.* Newport) characterized by resistance to at least ampicillin, cephalothin, chloramphenicol, clavulanic acid, streptomycin, sulfamethoxazole, tetracycline, and ceftriaxone (intermediate to complete) recently identified by the National Antimicrobial Resistance Monitoring System (NARMS), Centers for Disease Control and Prevention (CDC), (1) is an example of the emergent problem of antibiotic resistance. However, only a few state laboratories routinely perform susceptibility testing on enteric organisms, particularly in a format comparable to that employed by NARMS. Rapid identification of drug resistance is critical for preventing and treating diseases and for epidemiologic analysis (2).

Pulsed-field gel electrophoresis (PFGE) testing is more common at state public health laboratories; 50 such laboratories participate in CDC's PFGE surveillance program, PulseNet. In PulseNet, participants submit PFGE data on *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., and *Listeria monocytogenes* to a national database (3). Because PulseNet laboratories follow a standardized protocol, PFGE patterns can be compared reliably within the network and associations made promptly among isolates that may be few in number and widely separated geographically.

However, the clonal nature or inherent genetic variability of bacteria may limit the ability of PFGE either to link isolates or

to detect relatedness among a set of isolates from a single outbreak (4). In some organisms, such as *Shigella* spp. and *Campylobacter* spp., PFGE patterns vary considerably, and differences in banding patterns can occur among isolates that are epidemiologically linked (5). In other organisms, such as *Salmonella* serotype Enteritidis, variability of PFGE patterns is limited, and banding patterns can be indistinguishable among isolates that are not epidemiologically linked. Often, supplemental methods are needed to detect an association among isolates because of their apparent clonal characteristics (6). Therefore, PFGE databases must contain patterns from a sufficient number of isolates of a species representative of circulating strains to enable accurate interpretation of relatedness.

In Massachusetts, *S.* Newport isolates analyzed by PFGE have shown a high degree of variability. Ribotyping has been reported to be less discriminatory than PFGE (7) but can provide information that identifies real associations undetected solely by PFGE analysis, particularly when epidemiologic data are limited (8). We evaluated the usefulness of PFGE and automated ribotyping, independently and together, to monitor and characterize the MDR strain of *S.* Newport in Massachusetts.

## The Study

Specimens of *Salmonella* spp. submitted to the State Laboratory Institute as pure cultures or isolates from fresh stool samples submitted in Meridian Para-Pak C & S medium (Meridian Bioscience, Inc., Cincinnati, OH) were serotyped by the Kaufmann-White scheme, according to CDC protocols (9). After an increase in the incidence of *S.* Newport , the Massachusetts Department of Public Health initiated enhanced surveillance for this bacterium. In December 2000, we posted patterns MA-JJP0034 and MA-JJP0070 on the PulseNet listserv and asked other states to determine detection of *S.* Newport (CDC PulseNet patterns JJP.X01.0014 and JJP.X01.0181, respectively). In response, Oklahoma, Minnesota, Maine, and Vermont posted similar or indistinguishable isolates. In January 2001, *S.* Newport was isolated from a stool specimen from an employee at a Massachusetts farm that also reported diarrheal illness in cows. Investigators from the Massachusetts Department of Public Health and the Massachusetts Department of Food and Agriculture, Bureau of Animal Health, with assistance from CDC, visited farms and auction houses to obtain stool specimens from cows and calves exhibiting diarrheal illness. Isolates identified as *S.* Newport were tested by PFGE and automated ribotyping, and for antimicrobial resistance.

All *S.* Newport isolates were tested for resistance to amikacin, ampicillin, amoxicillin/clavulanic acid, apramycin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/sulphamethoxazole by using the Trek Diagnostics Sensititre CMV1UIL (Trek Diagnostic Systems, Inc., Cleveland, OH) plate. Isolates categorized as MDR *S.* Newport were resistant to at least ampicillin, cephalothin, chloramphenicol, clavulanic

*Massachusetts Department of Public Health, Jamaica Plain, Massachusetts, USA

acid, streptomycin, sulfamethoxazole, and tetracycline, and demonstrated complete or intermediate resistance to ceftriaxone. An *E. coli* strain, ATTC 25922, was used as a control organism during each susceptibility test run.

PFGE was performed on all *S.* Newport isolates, according to previously described methods (10). Automated ribotyping was performed by using the Riboprinter Microbial Characterization System (DuPont Qualicon, Wilmington, DE), according to the manufacturer's protocol. Briefly, a sweep of pure *S.* Newport cells from each test isolate was suspended in a 12- x 75-mm Falcon tube in sample buffer to 20% transmittance in a Vitek Colorimeter (bioMérieux, Durhan, NC). Thirty microliters of each suspension was transferred to one well in an eight-well sample carrier and heat treated to 80°C for 30 min; 5 µL of lysis buffers A and B was then added. The sample carrier was then placed in the Riboprinter for automated processing, which included the following steps: DNA extraction, *Pvu*II restriction and electrophoresis through a 1% agarose gel, DNA transfer, and hybridization to a nylon membrane. The DNA was probed with a sulfonated 6.7 Kb probe derived from the E. coli rRNA operon, which was detected with alkaline phosphatase–labeled anti-sulfonated anti-DNA antibodies. Ribotype images were captured by a charge-coupled device camera and compared for similarity to images in a *Pvu*II database of 272 *Salmonella* patterns of 138 serotypes (11).

PFGE patterns were analyzed with Molecular Analyst Software Version 1.11 (Bio-Rad Laboratories, Hercules, CA) by using the Dice coefficient with a band position tolerance of 1.5%. This method considers only the presence or absence of a band. All *S.* Newport patterns were compared with pattern MA-JJP0034, the first pattern of MDR *S.* Newport that appeared in Massachusetts. Patterns with a similarity coefficient of ≥0.85 relative to pattern MA-JJP0034 were designated in the analysis as MDR *S.* Newport by PFGE (D. Boxrud, pers. comm.).

Ribotype patterns were analyzed by the Riboprinter software, using the Pearson coefficient against a database of unique patterns of *Salmonella* serotypes. In this study, the *S.* Newport ribotypes with a similarity ≥0.85. were included in the MDR ribogroup. Sensitivity and specificity of the PFGE and ribotyping methods to identify MDR *S.* Newport were calculated and compared to the test results obtained by serotyping and antibiotic sensitivity testing for the 130 *S.* Newport isolates (12).

## Conclusions

One hundred sixteen *S.* Newport isolates from 300 human specimens and 14 *S.* Newport isolates from 50 bovine specimens were identified by serotyping at the State Laboratory Institute during the study period; 64 isolates were identified as MDR *S.* Newport (50 [43%] of 116 human isolates and all 14 bovine isolates). In addition to the eight-drug resistance pattern of the MDR isolates, 11 of 50 human isolates and 11 of 14 bovine isolates were resistant to kanamycin.

Two ribotype patterns, D-81 and D-82, designated as *S.* Newport in the Riboprinter database, were associated with the 130 *S.* Newport serotyped isolates (Figure 1). The 64 MDR *S.*
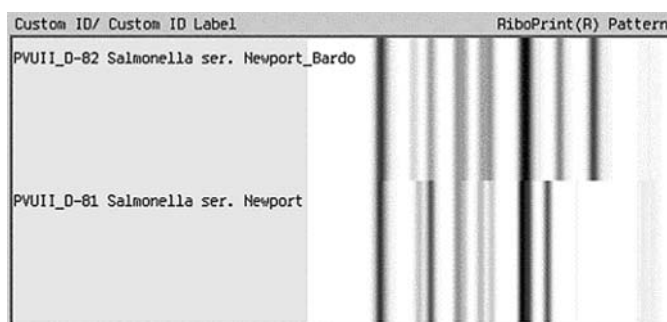


Figure 1. Ribotype patterns for *Salmonella* Newport.

Newport isolates, 50 human isolates, and 14 bovine isolates, matched only to ribotype pattern D-81. Sixteen of 66 pansusceptible *S.* Newport human isolates also were identified as D-81 ribotype pattern. The remaining 50 pansusceptible *S.* Newport isolates were identified as D-82 ribotype pattern (Table). Using pattern D-81, ribotyping had a sensitivity of 100% and a specificity of 76% in identifying MDR *S.* Newport among *S.* Newport isolates.

Among the 130 human and bovine *S.* Newport isolates were 58 PFGE patterns of *Xba*I digests (Figure 2). Eight PFGE patterns were associated with 64 MDR *S.* Newport isolates (Figure 3). Two of the 66 pansusceptible isolates smeared by PFGE were not interpretable, leaving 64 isolates for PFGE analysis. The most common PFGE pattern was MA-JJP0034, which appeared in 41 (64%) of 64 of the MDR *S.* Newport isolates. MA-JJP00015 was the most common pattern among the pansusceptible isolates, being found in 7 (11%) of 64 isolates. When the *S.* Newport isolates were analyzed against PFGE pattern 34, all MDR *S.* Newport isolates and 7 of 64 pansusceptible *S.* Newport isolates had a similarity coefficient of >0.85. Thus, PFGE had a sensitivity of 98% and a specificity of 89% when this pattern was used to screen this set of isolates.

The 64 MDR *S.* Newport, among a sample of 130 *S.* Newport isolates, were correctly identified by comparing the sensitivity and specificity of PFGE and automated ribotyping to conventional serotyping and antibiotic susceptibility test results. The sensitivity of each method was ≥98%. The specificity of PFGE and automated ribotyping was 89% and 76%, respectively, with no false-negative results, and 11% and 24%, respectively, of *S.* Newport isolates misidentified (false positive) as the MDR strain.

Automated ribotyping was a rapid means of subtyping *S.* Newport and distinguishing the MDR strain from non-MDR strains by using the Riboprinter *Pvu*II database. This capability is important because most often *Salmonella* isolates are submitted to state laboratories and characterized to the O antigen level, not the species level. Further, serotyping performed at a state public health laboratory to define the *Salmonella* species requires a minimum of 2 days, and for biphasic *Salmonella* species, such as *S.* Newport a minimum of 4 days (9). PFGE was useful in distinguishing associations within the isolates of *S.* Newport*,* which allowed for identification of potential epidemiologically related events, such as the PFGE pattern of

Table. Sensitivity and specificity of PFGE versus. ribotyping in identifying MDR *Salmonella* Newport[a]

| | PFGE of *S.* Newport | | | Ribotyping of *S.* Newport | | | |
|---|---|---|---|---|---|---|---|
| PFGE pattern MA-JJP0034 | MDR | Not MDR | Total | D-81 ribotype | MDR | Not MDR | Total |
| Yes[b] | 63 | 7 | 70 | Yes | 64 | 16 | 80 |
| No | 1 | 57 | 58 | No | 0 | 50 | 50 |
| | 64 | 64[c] | 128 | | 64 | 66 | 130 |

[a]PFGE, pulsed-field gel electrophoresis; MDR, multidrug resistant.
[b]>85% relatedness to MA-JJP0034.
[c]Two pansusceptible isolates smeared by PFGE.

MDR *S.* Newport common among a subset of bovine and human isolates. After a pure culture isolate is obtained, turnaround times for PFGE and ribotyping testing are approximately 24 h and 8 h, respectively; however, serotype identification often is required for interpretation of PFGE test results, depending on the organism, which can lengthen turnaround for final results by 1 or more days.

Both automated ribotyping and PFGE rely on existing databases of images from individual isolates to characterize DNA fingerprints. As data from more strains are added to databases, characterizing similarities or differences between isolates
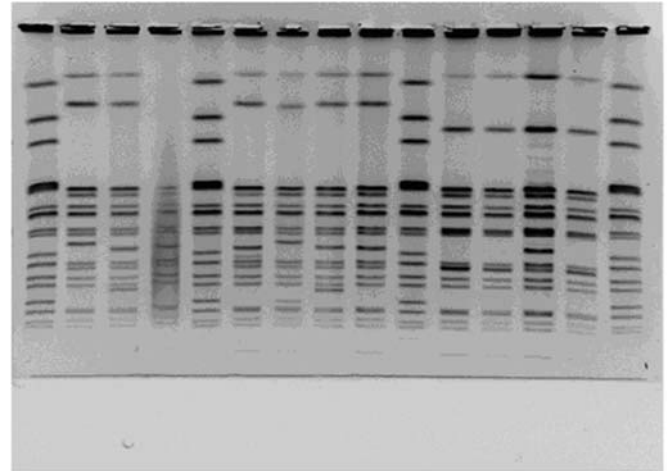


Figure 3. Pulsed-field gel electrophoresis patterns of XbaI-digested multidrug-resistant *Salmonella* Newport. Lane 2, pattern MA-JJP0036; lane 3, pattern MA-JJP 0027; lane 6, pattern MA-JJP0062; lane 7, pattern MA-JJP0077; lane 8, pattern MA-JJP0089; lane 9, pattern MA-JJP0034; lane 11, pattern MA-JJP0050; lane 13, pattern MA-JJP0070.

becomes easier. Because the fingerprint database of the PulseNet program is extensive and PFGE is more discriminatory that ribotyping, PFGE provides a more robust tool in characterizing the development of emerging pathogens.

These data suggest that PFGE and ribotyping can be used together to provide rapid identification of the MDR strain of *S.* Newport. These methods offer important capabilities for laboratory-based surveillance for public health purposes; however, automated ribotyping is very costly and should be used in a public health laboratory only if justified by appropriate cost-benefit analysis. Furthermore, these data support the need for strengthening public health laboratory infrastructure to facilitate early detection of infectious disease risks.
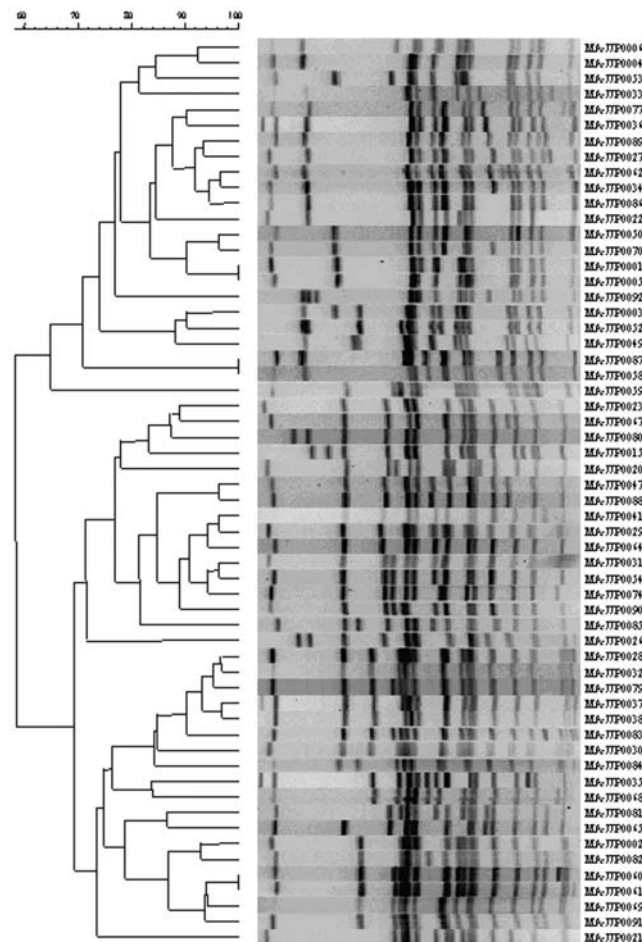


Figure 2. Dendrogram of unique pulsed-field gel electrophoresis patterns of *Salmonella* Newport. The 58 patterns represent all patterns received at the State Laboratory Institute during April 1999–April 2001. * indicates multidrug-resistant *S.* Newport patterns.

Dr. Fontana is director of the Pulsed Field Gel Electrophoresis and Antimicrobial Resistance Laboratory at the Massachusetts Department of Public Health/State Laboratory Institute. With the support of the State Laboratory Institute and epidemiologists in the Division of Communicable Disease, Dr. Fontana established the Antimicrobial Resistance Surveillance Laboratory in 1999, which performs susceptibility testing on invasive *Streptococcus pneumoniae*, vancomycin resistant enterococci, *Salmonella* spp., *Shigella* spp., and *Escherichia coli* O157:H7.

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Address for correspondence: John Fontana, Massachusetts Department of Public Health, State Laboratory Institute, 305 South Street, Jamaica Plain, MA 020130, USA; fax: 617-983-6618; email: john.fontana@state.ma.us

# Class I Integrons and SXT Elements in El Tor Strains Isolated before and after 1992 *Vibrio cholerae* O139 Outbreak, Calcutta, India

**Amita,\* S. Roy Chowdhury,\* M. Thungapathra,\* T. Ramamurthy,† G. Balakrish Nair,†‡ and Amit Ghosh\***

We examined the distribution of class I integrons and SXT elements in *Vibrio cholerae* O1 El Tor strains, isolated in Calcutta, India, before and after the *V. cholerae* O139 outbreak in 1992. Class I integrons, with *aadA1* gene cassette, were detected primarily in the pre-O139 strains; the SXT element was found mainly in the post-O139 strains.

Since the introduction of antibiotics in the treatment of infectious diseases, antibiotic resistance has spread dramatically among microbes. The occurrence of drug-resistant strains of *Vibrio cholerae* is being reported with increasing frequency (1). Spread of antibiotic resistance in microbes has been attributed to the mobilization of drug-resistance markers by a variety of agents (e.g., plasmids, transposons, and integrons). In *V. cholerae*, antibiotic-resistance determinants have traditionally been found on plasmids. Recently, in a few cases, these determinants have also been detected on integrons and a novel conjugative transposable element, SXT.

Integrons are DNA elements capable of mobilizing individual gene cassettes into bacterial chromosomes by site-specific recombination. Integrons consist of a central variable region that often harbors antibiotic-resistance gene cassettes, flanked by 5′ and 3′ conserved sequences (CS) (2). Integrons have been categorized into four different classes on the basis of the distinctive integrase (*int*) genes they carry on their 5′-CS (2,3). Among the different integron families, class I integrons are found to be most prevalent in drug-resistant bacteria. Class I integrons have been detected in *V. cholerae* O1 strains isolated in Vietnam, Thailand, and Italy (4–6). Their presence in *V. cholerae* O1 strains isolated in India, however, had not been previously reported. SXT is a transmissible genetic element that harbors resistant determinants to trimethoprim, streptomycin, sulfamethoxazole, and chloramphenicol. SXT was first discovered in *V. cholerae* O139 (7), a new epidemic strain that

*Institute of Microbial Technology, Chandigarh, India; †National Institute of Cholera and Enteric Diseases, Calcutta, India; and ‡International Centre for Diarrheal Disease Research, Dhaka 1000, Bangladesh

emerged in the Indian subcontinent in late 1992 and displaced the *V. cholerae* O1 El Tor as the primary cholera-causing agent for approximately 6 months. After this period, *V. cholerae* O1 El Tor reemerged and became predominant (8). Unlike those of the pre-O139 period, most of these poststrains were found to be resistant to trimethoprim, sulfamethoxazole, and streptomycin, and in a few cases, this resistance was found to be due to the SXT element. We describe the results of a study in which we examined, retrospectively, the presence and the relative abundance of class I integrons and SXT elements in *V. cholerae* El Tor O1 strains, isolated in Calcutta, before, during, and after the O139 outbreak.

## The Study

A total of 58 strains of *V. cholerae* O1 El Tor isolated in Calcutta before (March–December 1992; group I), during (July–November 1993; group II), and after (March 1994–June 1995; group III) the *V. cholerae* O139 outbreak (8) were included in this study. These strains, belonging to ribotypes RI, RII, and RIII, were maintained in brain-heart infusion broth supplemented with 15% glycerol at –70°C and grown when needed (8).

### Occurrence of Class I Integrons

The 3′ conserved sequence of class I integrons is characterized by antibiotic resistance gene *qacEΔ1* and the sulfonamide resistance gene *sul1*. To identify class I integrons, primers ATCGCAATAGTTGGCGAAGT (accession no. X15370) and GCAAGGCGGAAACCCGCGCC (accession no. X12869), specific for 3′ CS (5), were amplified in this region by polymerase chain reaction as described (5). A 0.8-kb product, found in 22 of the 58 isolates, was confirmed to be the 3′ CS of class I integrons by sequencing (Table). To identify the gene cassette in the class I integrons, primers previously used to amplify the region between the 5′ CS and 3′ CS (5) were used. All 22 strains produced a 1.0-kb amplicon, which on sequencing was found to contain the *aadA1* gene that confers resistance against aminoglycosides, streptomycin, and spectinomycin (4,5). Nucleotide sequence of *aadA1* cassette has been assigned the GenBank accession no. AY115577.

A total of 17 out of the 22 class I integron-bearing strains belonged to the before period. The remaining five, though isolated after June 1993, probably represented carry-over strains. These five strains belonged to the same RI ribotype as the other 17 strains from the before period (Table) and had a CTX structure identical to the other strains with RI ribotype (8).

### Antibiotic Resistance Profile

Resistance of *V. cholerae* isolates to ampicillin (10 µg), ciprofloxacin (5 µg), furazolidone (50 µg), gentamycin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulfamethizole (100 µg), tetracycline (30 µg), and trimethoprim (25 µg) were examined by using commercial discs (Hi Media, Bombay, India) as described (1). An overall increase in the number of strains with resistance to a greater

Table. Class I integron and SXT profiles of 58 *Vibrio cholerae* strains isolated before, during, and after the 1993, *V. cholerae* O139 outbreak, Calcutta

| Group | Strain no. | Ribotype[a] | Class I integron | SXT | Antibiogram[b] |
|---|---|---|---|---|---|
| I | VC1 | RI | + | | A, N, S, Sm |
| | VC2 | RI | + | | Fz, S, Sm |
| | VC3 | RII | | | Fz, Sm |
| | VC5 | RI | + | | A, Fz, S, Sm |
| | VC12 | RI | + | | N, S, Sm |
| | VC14 | RI | | + | S, Sm, Tr |
| | VC20 | RI | + | | N, S, Tr |
| | VC35 | RI | | | Fz, N |
| | VC41 | RI | | | A, Fz, N |
| | VC44 | RI | + | | A, Fz, N, S, Sm |
| | VC48 | RI | + | | A, Fz, N, S, Sm |
| | VC54 | RI | + | | A, N, S, Sm |
| | VC55 | RI | + | | Fz, N, S, Sm |
| | VC59 | RI | + | | A, N, S, Sm |
| | VC70 | RI | + | | Fz, S, Sm |
| | VC72 | RI | + | | S, Sm |
| | VC73 | RI | + | | S, Sm |
| | VC80 | RI | + | | S, Sm |
| | VC99 | RI | + | + | A, Fz, N, S, Sm, Tr |
| | VC100 | RI | + | + | A, Fz, N, S, Sm, Tr |
| | VC105 | RI | | + | S, Sm, Tr |
| | VC106 | RI | + | | N, S |
| II | CO222 | RI | + | | Fz, S, Sm |
| | CO327 | RIII | | + | A, N, S, Sm, Tr |
| | CO334 | RI | + | + | A, N, S, Sm, Tr |
| | CO366 | RIII | | + | A, S, Sm, Tr |
| | CO370 | RIII | | + | S, Sm, Tr |
| | CO371 | RI | + | | S, N |
| | CO374 | RIII | | + | A, N, S, Sm, Tr |
| | CO387 | RIII | | + | N, S, Sm, Tr |
| | CO394 | RIII | | + | A, Fz, S, Sm, Tr |
| | CO407 | RIII | | + | A, Fz, N, S, Sm, Tr |
| | CO416 | RIII | | + | A, N, S, Sm, Tr |
| | CO417 | RIII | | + | A, N, S, Sm, Tr |
| | CO423 | RIII | | + | A, N, S, Sm, Tr |
| | CO424 | RIII | | + | A, Fz, N, S, Sm, Tr |
| | CO427 | RIII | | + | A, Fz, N, S, Sm, Tr |
| III | CO458 | RI | + | + | N, S, Sm, T, Tr |
| | CO459 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO460 | RIII | | + | Fz, Na, S, Sm, Tr |
| | CO461 | RI | + | + | N, S, Sm, T, Tr |
| | CO471 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO473 | RIII | | + | Fz, Na, S, Sm, Tr |
| | CO474 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO475 | RIII | | + | A, Fz, S, Sm, Tr |
| | CO580 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO650 | RIII | | + | A, Fz, N, S, Sm, Tr |
| | CO720 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO770 | RIII | | + | A, Fz, Na, N, S, Sm, Tr |
| | CO810 | RIII | | + | Fz, Na, N, S, Sm, Tr |
| | CO825 | RIII | | + | A, Fz, Na, N, S, Sm, Tr |
| | CO835 | RIII | | + | Fz, Na, S, Sm, Tr |
| | CO839 | RIII | | + | A, Fz, Na, N, S, Sm, Tr |
| | CO840 | RIII | | + | Fz, N, S, Sm, Tr |
| | CO860 | RIII | | + | A, Fz, N, S, Sm, Tr |
| | CO910 | RIII | | + | Fz, N, S, Sm, Tr |
| | CO950 | RIII | | + | A, Fz, N, S, Sm, Tr |
| | CO970 | RIII | | + | A, Fz, Na, N, S, Sm, Tr |

[a]Sharma et al. (8).
[b]A, ampicillin; Cf, ciprofloxacin; Fz, furazolidone; G, gentamycin; N, neomycin; Na, nalidixic acid; S, streptomycin; Sm, sulfamethizole; T, tetracycline; Tr, trimethoprim

number of antibiotics was seen in the isolates of the post-O139 period; a fourfold increase in resistance against trimethoprim, a drug often used for the treatment of cholera in children and pregnant women, was noted. Almost all strains, from all isola-

tion periods, were uniformly resistant to streptomycin and sulfamethizole, but none were found to be resistant to tetracycline, gentamycin, or ciprofloxacin. None of the strains examined were found to harbor any plasmid.

## Presence of SXT Elements

None of the strains belonging to the ribotypes RII and RIII, which were all isolated during and after the O139 outbreak, carried the *aad*A1 gene cassette (coding for aminoglycoside resistance); however, they were all resistant to streptomycin. Further, all of the strains were resistant to trimethoprim. Since strains of *V. cholerae,* which harbor the SXT element, are resistant to streptomycin and trimethoprim (7), we sought to determine if the resistance of the post-O139 strains to streptomycin and trimethoprim could be traced to an SXT element. Colony hybridization of the 58 strains with a 0.8-kb probe, specific for the SXT integrase gene (7), showed that all trimethoprim-resistant strains from all isolation periods and all "during" and after streptomycin-resistant strains carried the SXT element (Table). Further, five of these strains harbored both SXT and the *aad*A1 gene cassette (Table). Our survey thus suggested that while the streptomycin resistance of the strains isolated before the O139 outbreak was due to the *aad*A1 gene cassette carried by the class I integrons, the SXT element was probably responsible for this phenotype in the post-O139 strains.

All *V. cholerae* strains included in this study were resistant to multiple antibiotics. Since none of the strains harbored any plasmid and the resistance determinants present in the class I integrons and in the SXT element could account for only a few markers, other determinants of antibiotic resistance may exist.

Dalsgaard et al. (4) found that the O1 strains isolated in Vietnam in 1994 and after, had the ribotype identical to that found in some of the O1 strains isolated in Samutsakorn, Thailand, during and after the 1993 O139 outbreak there. Both pre- and post-O139 isolates carried class I integrons with the *aadA2* gene cassette. This fact led Dalsgaard et al. to conjecture that this distinct O1 strain might have been transferred between Thailand and Vietnam (5). However, the possibility of its migrating from a third country could not be ruled out. It should be noted that the ribotype of the pre-O139, O1 Calcutta strains examined in this study was identical to that of the Samutsakorn strains (4,5,8). However, as can be seen from the data presented here, unlike the Samutsakorn strains, these strains harbored *aadA1* gene cassette.

We had shown previously that the post-O139, O1 strains isolated in Calcutta could have migrated to Guinea-Bissau in Africa (9). Further evidence has shown that this strain, which caused an outbreak in 1994–1995, could have subsequently acquired class I integrons bearing the [*ant(3")-1a*] gene cassette by a 150-kb plasmid from an unknown source (10).

## Conclusions

While the class I integron with *aadA1* gene cassette was widely distributed among the pre-O139 O1 strains isolated in Calcutta, it was mostly absent in the post-O139 O1 strains
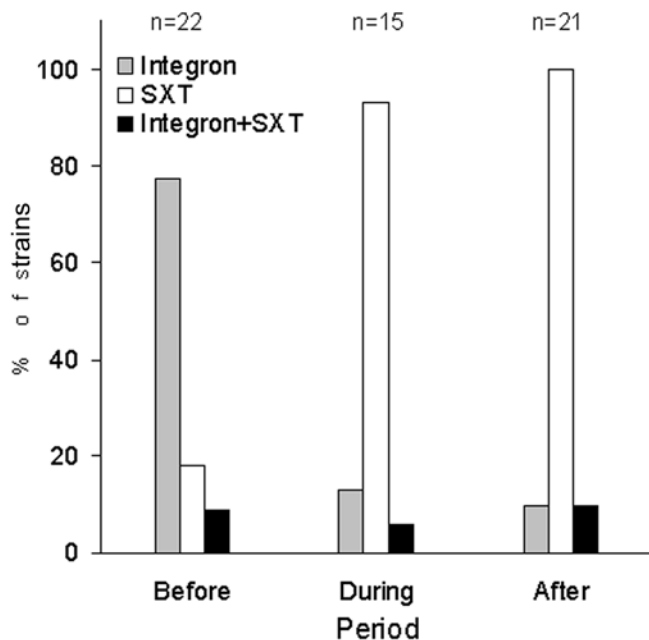
n=22    n=15    n=21

Figure. Distribution of class I integrons and SXT elements in Vibrio *cholerae* El Tor strains isolated before, during, and after the O139 outbreak.

(Figure). This finding is in contrast to other studies involving the SXT element; 80% of all pre-O139 strains were devoid of SXT, whereas all post-O139 strains (with the exception of a few carry-over strains) had it (Figure). When the data presented in this paper are considered together with the information presented by others (4,5,8,10), it appears that both pre- and post-O139, O1 strains, isolated in Calcutta, probably could have moved to other countries and became established there.

Ms. Amita holds a master's degree in microbiology and is working towards her doctoral degree under the supervision of Dr. Amit Ghosh. She is researching the emergence of drug resistance in *Vibrio cholerae*.

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Address for correspondence: Amit Ghosh, Institute of Microbial Technology, Sector 39A, Chandigarh 160036, India; fax: 91-172-690585/690632; email: director@imtech.res.in

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# Fear of Bioterrorism and Implications for Public Health Preparedness

**Mark S. Dworkin,\* Xinfang Ma,\***
**and Roman G. Golash\***

After the human anthrax cases and exposures in 2001, the Illinois Department of Public Health received an increasing number of environmental and human samples (1,496 environmental submissions, all negative for *Bacillus anthracis*). These data demonstrate increased volume of submissions to a public health laboratory resulting from fear of bioterrorism.

After the terrorist attacks of September 11, 2001, and the discovery of human anthrax cases and exposures several weeks later on the East Coast, Illinois Department of Public Health's (IDPH) Division of Laboratories received environmental and human samples for analysis as part of suspected bioterrorism investigations. The large number of samples provided an opportunity to learn about the possible presence of *Bacillus anthracis* in the environment, to gain insight into the frequency of true versus perceived bioterrorist events, and to observe and respond to the impact of widely publicized terrorism alerts.

## The Study

Submissions of powder and nonpowder environmental samples and human blood or tissue specimens submitted from October 8 through December 31, 2001, were reviewed. Before samples were accepted by IDPH Division of Laboratories, incidents involving environmental samples had to be reviewed by Federal Bureau of Investigations (FBI) agents who determined if a potential bioterrorism threat was credible. The IDPH laboratories in Chicago and Springfield processed all samples submitted through law enforcement authorities in this manner. The Chicago laboratory received samples primarily from northern Illinois (north of Interstate 80), while the Springfield laboratory received samples from central and southern Illinois. The Chicago laboratory followed all required guidelines for a biosafety level 3 laboratory (1).

The laboratory methods for identifying *Bacillus* species in environmental samples have been reported elsewhere (2). Laboratory methods included Gram stain and culture of suspicious colonies grown on blood agar plates, and beta-lactamase, motility, and gamma-phage lysis testing. A malachite green stain for spores was performed on all powder specimens during the initial 2 weeks at the Chicago laboratory and on selected

*Illinois Department of Public Health, Chicago and Springfield, Illinois, USA

specimens thereafter; an M'Fadyean stain was used at the Springfield laboratory. Polymerase chain reaction (PCR) was performed on those samples requiring the most rapid turnaround time (e.g., specimens submitted by a U.S. Postal Service facility that had been closed pending results). Human samples arrived from hospital laboratories in the form of a tryptic soy agar slant and were plated to blood agar plates upon arrival. Gamma-phage lysis, PCR, or both were performed as needed. The Chicago and Springfield laboratories' processed their first samples on October 8 and October 9, respectively.

Because no data were available regarding what to expect from processing bioterrorism threat-related samples from the environment, *Bacillus* organisms from most environmental and human samples were speciated, even if negative for *B. anthracis*.

### Environmental Specimens

A total of 1,496 environmental specimens were processed: 1,193 (79.7%) in Chicago and 303 (20.3%) in Springfield. An additional 40 human specimens were processed, 28 (70%) in Chicago and 12 (30%) in Springfield. Chicago sample submissions rose steadily after the first week of October and peaked during the week of October 29 through November 4, with the largest number of submissions processed on November 7 (range 0–64 submissions per day) (Figure). An additional 17 submissions for which the date of submission was not clearly documented, and may have preceded October 8, also were processed. Powdery substances constituted 42.0% of submissions to the Chicago laboratory versus 33.7% of submissions to the Springfield laboratory. Nonpowdery substances (e.g., environmental swab samples, letters, envelopes, packages, and other materials) constituted 58.0% of submissions to the Chicago laboratory versus 66.3% of submissions to the Springfield laboratory. Eight additional environmental samples
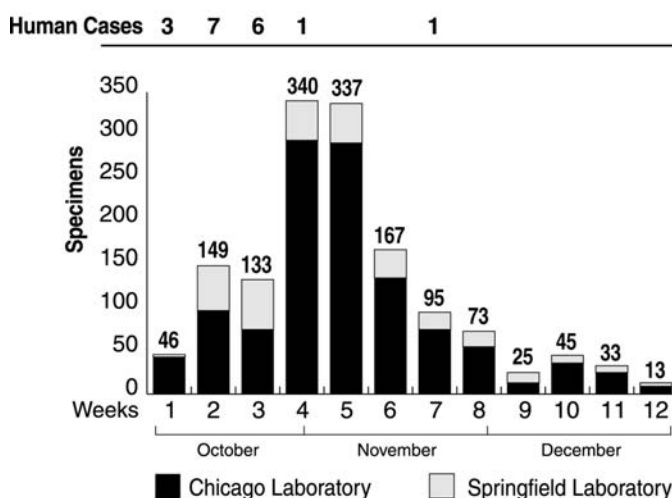


Figure. Number of environmental specimens submitted to the Illinois Department of Public Health Division of Laboratories for *Bacillus anthracis* testing each week from October 8 through December 30, 2001 and number of human cases occurring on the East Coast and reported each week in the news media.

that did not go through the FBI were received by the Chicago laboratory from hospitals.

Among both the powders and nonpowders processed at the Chicago laboratory, the most frequently isolated organisms were *Bacillus cereus* (19.2% and 8%, respectively) and non-hemolytic staphylococci (23.9% and 22.8%, respectively) (Table 1). Among the eight hospital samples not submitted through the FBI, *B. megaterium* (four), *B. thuringiensis* (one), and nonanthracis *Bacillus* species (three) were identified. Twenty specimens (8 in Chicago and 12 in Springfield) were processed by using PCR. All of these results were negative for *B. anthracis*.

A review of the source and circumstances related to 57 non-human samples submitted to the Chicago laboratory on November 7, 2001 (the date with the highest number of sub-missions), indicated that most of the items were mail items (e.g., 18 letters or envelopes, 12 unspecified mail items, and 7 packages), but powders (7 submissions) and unspecified "sus-picious substance" (13 items) also were identified. Such items came from at least six counties and involved 13 local police departments, the Illinois State Police, two fire departments, and the FBI. In addition to submissions from private citizens, sub-missions came from two universities, a post office, a private business, and a consulate. A high level of anxiety among the public was likely responsible for the otherwise unsuspicious items submitted, including dairy creamer, powder from donuts, a backpack, a telephone, a frozen dinner, a computer keyboard, and a letter from a married man's lover that was intercepted by his wife and submitted unopened as a suspicious mail item.

## Human Specimens

Twenty-eight human specimens were submitted to the Chicago state laboratory for evaluation after preliminary test-ing at an initial laboratory (usually a hospital) could not rule out *B. anthracis* (Table 2). These included 15 blood cultures.

For 12 specimens, the species were not identified, although test results demonstrated they were not any of 16 *Bacillus* species. An additional 12 specimens submitted to the Springfield labo-ratory were negative for *B. anthracis*.

## Conclusions

We found no samples positive for *B. anthracis* among the nearly 1,500 submissions to IDPH for testing. Other state health departments also received large numbers of submis-sions: Michigan and Oklahoma received at least 228 and 762 submissions, respectively (3,4). These results demonstrate the potential stress to a public health laboratory that may result from bioterrorism-related anxiety and hyperalertness to the environment. This high state of alert by the public is important for early recognition of a real bioterrorism event through enhanced reporting.

Our data demonstrate that the number of submissions was temporally associated with the media attention to anthrax-relat-ed events in Florida, New York City, Washington, D.C., and other affected areas. Concern was reinforced with messages from government, warning of the need for the public to be on heightened alert for terrorism. On October 29, 2001, the U.S. attorney general and the FBI director announced that U.S. citi-zens and law enforcement agencies should be on "highest alert" based on "credible" information, and police and citizens should be "extremely vigilant." Forty-five percent of the nearly 1,500 environmental specimens submitted to the state laboratory for testing during the 12-week period arrived during the 2 weeks after that announcement, which created an unprecedented workload in bioterrorism evaluation.

During the surge in laboratory demand, adequate numbers of trained personnel to process the large volume of submissions were needed. Additional staff were trained, and work hours were expanded. Despite these efforts, a backlog occurred, caus-ing specimens to be grouped in order of priority for rapid ver-

Table 1. Results of 1,364 environmental specimens submitted to the Illinois Department of Public Health Division of Laboratories (Chicago laboratory) for *Bacillus anthracis* testing, October–December 2001

| Organism | Powder (%) | Nonpowder (%) |
|---|---|---|
| Total | 573 (42.0) | 791 (58.0) |
| *B. cereus* | 110 (19.2) | 63 (8.0) |
| *B. subtilis* | 29 (5.1) | 32 (4.0) |
| Other *Bacillus* species (hemolytic, positive motility) | 21 (3.7) | 40 |
| *B. mycoides* | 16 (2.8) | 6 |
| *B. circulans* | 10 (1.7) | 4 |
| *B. pumilus* | 2 (0.3) | 5 |
| *B. brevis* | 1 (0.2) | 1 |
| *B. laterosporus* | 0 | 1 |
| *B. megaterium* | 0 | 1 |
| *B. polymyxa* | 0 | 1 |
| *B. anthracis* | 0 | 0 |
| *Staphylococcus* species (nonhemolytic) | 137 (23.9) | 180 |
| *Staphylococcus aureus* | 3 (0.5) | 5 |
| Other mixed gram-positive organisms | 48 (8.3) | 68 |
| Gram-negative bacilli | 80 (14.0) | 72 |
| *Enterobacter agglomerans* | 1 (0.2) | 0 |
| *Moraxella catarrhalis* | 1 (0.2) | 0 |
| Mixed other gram-positive organisms and mold | 8 (1.4) | 11 |
| Mold | 19 (3.3) | 32 |
| No growth | 87 (15.2) | 269 |

Table 2. Results of 28 human specimens submitted to the Illinois Department of Public Health Division of Laboratories (Chicago laboratory) for *Bacillus anthracis* testing, October–December 2001[a]

| Organism | No. (%) | Source (no.) |
|---|---|---|
| *B. cereus* | 5 (17.9) | Blood (3), nasal (2) |
| *B. megaterium* | 4 (14.3) | Blood (2), leg wound (1), abdominal fluid (1) |
| *B. subtilis* | 2 (7.1) | Blood (2) |
| *B. brevis* | 1 (3.6) | Wound (1) |
| *B. coagulans* | 1 (3.6) | Blood (1) |
| *B. firmus* | 1 (3.6) | Blood (1) |
| *B. pumulus* | 1 (3.6) | Blood (1) |
| *Paenibacillus macerans* | 1 (3.6) | Unspecified |
| *Bacillus* species, other (not speciated) | 12 (42.9) | Blood (8), nasal (1), body fluid (1), cerebrospinal fluid (1), unspecified (1) |
| *B. anthracis* | — | — |

[a]After preliminary testing at an Illinois laboratory could not rule out *B. anthracis*.

sus delayed processing. Delay in processing added to the anxiety experienced by many who submitted samples. Laboratory staff were distracted by frequent requests for updated information. Because of evolving information, emerging guidance from the Centers for Disease Control and Prevention, and the difficulty of managing staff who were being pulled away from other laboratory jobs to assist with bioterrorism samples, meetings were held frequently (often more than once per day). However, such meetings also competed with laboratory time, which was needed to process the increasing load of submissions.

Among the more positive aspects of the high volume of submissions was enhanced cooperation with law enforcement officials, especially the FBI. Such officials maintained a temporary office in the Chicago laboratory facility, where submissions could be received at all hours, prioritized, and then delivered individually or batched for submission to the laboratory (which did not have a night shift of workers) during working hours.

Because health department laboratories have previously had only limited and sporadic experience with the test methods involved in urgent microbiologic evaluation for anthrax, the experience with these submissions provided an opportunity to polish critical laboratory skills, to utilize infrequently used or not previously available equipment (e.g., real time PCR), to speciate nonanthracis *Bacillus* isolates to determine what species may be expected during such an event, and to anticipate what personnel and changes in protocols might be needed during future bioterrorist events. This experience also highlighted the importance of regular training and updating of laboratory staff on procedures relevant to bioterrorism agent evaluation.

We recommend continued close communication and collaboration between public health and law enforcement officials, which include developing flexible criteria on specimen submission guidelines for public laboratories. Development of standardized forms for information collection related to suspicious substance submissions may be useful in evaluating the epidemiology of any future bioterrorism events. We also recommend regular training of laboratory staff on procedures relevant

to bioterrorism agent evaluation, including cross-training of selected staff not usually involved in the handling of suspected bioterrorism agents as part of a surge capacity plan. Such a plan should also include a mechanism for handling submissions after regular work hours because of the high profile and expectations of turnaround time that submissions for bioterrorism agent evaluation usually have. Finally, enhancing communication between the many agencies involved remains both a challenge and an opportunity for future efforts to combat these kinds of events.

Dr. Dworkin is the state epidemiologist and team leader for the Rapid Response Team at the Illinois Department of Public Health. He is active in outbreak investigation, evaluation of infectious disease surveillence data, and epidemiologic issues related to bioterrorism.

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Address for correspondence: Mark S. Dworkin, State Epidemiologist for Infectious Diseases, Illinois Department of Public Health, 160 N. LaSalle, #7 South, Chicago, IL 60601, USA; fax: 312-814-4844; email: mdworkin @idph.state.il.us

# Cicero and *Burkholderia cepacia:* What's in a Name?

John E. Moore* and Frederick Williams†

> Then said they unto him, Say now Shibboleth: and he said Sibboleth: for he could not frame to pronounce it right. Then they took him and slew him, at the passes of Jordan: and there fell at that time of the Ephraimites forty and two thousand.
>
> Judges 12:6

In Old Testament times, mispronunciation bore a price. The Gileadites (circa 1143 B.C.) used pronunciation to differentiate their own from the Ephraimites, and the consequences of mispronunciation were severe. Today, mispronunciation, though not a matter of life and death, presents problems when it interferes with communication. In scientific nomenclature, Greek or Latin bionomials of infectious disease microorganisms are often mispronounced, sometimes causing confusion among healthcare professionals (e.g., infectious disease physicians, epidemiologists, and even microbiologists). Unlike horticulturalists, who have masterfully developed a large repertoire of common names for botanical species thereby avoiding the need for and potential mispronunciation of classical Greek and Latin, infectious disease specialists still rely on Greek and Latin bionomials.

How important is a standard pronunciation of bionomials? Language is about communication. Provided the parties in a discussion can understand each other, variations in pronunciation of individual words can be tolerated or disregarded. Everyday modern English is filled with examples of variant pronunciations that cause no communication problems (e.g., either, tomato, laboratory, fertile). These variant pronunciations have many causes. Regional practice is probably the single most important variant, but educational and social backgrounds also play a part, as do personal preferences and even etymologic theories. It would be futile and (some believe) undesirable to impose uniformity by prescribing approved pronunciations when communication is not compromised. Moreover, in all languages, pronunciation changes constantly.

*Burkholderia cepacia*, an important gram-negative bacterial pathogen in patients with cystic fibrosis, may cause premature death in these patients. Since its first description in 1950 by Walter Burkholder (1), the pathogen has undergone several taxonomic reclassifications (2) in accordance with the Bacteriologic Code (1990 revision). However, uncertainty still surrounds the clinical relevance of its evolving taxonomy, par-ticularly in regards to the nine described genomovars of the *B. cepacia* complex (BCC). The species name *cepa'cia* comes from L. fem. N. *caepa* or *cepa* (onion). Most confusion surrounding this species name was initially due to its transfer from the genus *Pseudomonas* to the newly described genus *Burkholderia* by Yabuuchi et al. in 1992 (3). The practice of renaming individual BBC genomovars with species names when phenotypic differentiation becomes available has heightened the confusion, as in the renaming of *B. cepacia* genomovar II to *B. multivorans,* where problems arise both with physicians (in infection control) and with patients (psychological acceptance of the disease).

Even though in *B. cepacia*, taxonomic issues rather than pronunciation are at the root of confusion, the pathogen neatly encapsulates several aspects of the linguistic conundrum involving "correct" pronunciation of Latin binomials. The correct pronunciation of both the genus *Burkholderia* and the species *cepacia* is still debated. The debate is mainly about the correct pronunciation of *cepacia*, but the genus name, *Burkholderia*, also deserves some consideration. The genus name is formed from the surname Burkholder, on which a Latin suffix *–ia* has been grafted. How should this synthetic word be pronounced? With the original pronunciation of the name retained as far as possible (long *o* and stress on second syllable) or with a more Latinized effect (short *o* and stress on third syllable, possibly lengthening its vowel)? Or is this in fact a nonexistent problem because the word is normally encountered in print so variations in pronunciation present no confusion?

The scientific and infectious disease communities would benefit from the adoption of a standard pronunciation of Latin binomials that would obviate confusion and ambiguities. The Linnaean binomial system uses Latin morphology and grammar in forming its names, and they are equally respected in China and Peru. Why not adopt a standard pronunciation? An immediate practical objection is that there is hardly a "standard Latin pronunciation." Throughout history, Latin pronunciation has developed in accordance to the vernacular language of its users. Even as long ago as the 16th century, when Latin was of necessity the common language of such multinational organizations as the Roman Empire and the Catholic Church, speakers of Latin from different nations could not understand each other. This linguistic situation was satirized in 1528 by Erasmus, who proposed to standardize a reconstructed version of classical Latin pronunciation, i.e., the practice (as far as it could be deduced) of 1,500 years earlier. His efforts had only

*Belfast City Hospital, Belfast, Northern Ireland; and †The Queen's University of Belfast, Belfast, Northern Ireland

limited success. Two and a half centuries later, Samuel Johnson, in his Life of Milton, condemned those who, like Milton, sought to replace the "English" pronunciation with the "Italian." A remnant of Johnson's "English" system still persists in the Latin-derived jargon used by British lawyers. Toward the end of the 19th century, schoolmasters and classics scholars began adopting a restored pronunciation (reconstructed from heterogeneous evidence) that aimed to reproduce Latin pronunciation in the time of Cicero or Virgil (i.e., the first centuries B.C. and A.D.). This reform was supported in 1923 by a committee appointed by the Prime Minister of the United Kingdom, but the "new way" was not universally accepted.

Analogous situations are found in other European countries. In Italy, the church pronunciation still carries much prestige. In France, the reform movement encountered bitter opposition. However, the views of responsible classics scholars today seem to converge in both theoretical and practical terms. The most promising system is described by W. Sidney Allen (4) in which he uses symbols of the International Phonetic Alphabet. According to this system, both *c*s in *cepacia* would be pronounced hard (like English *k*); the first vowel, *e*, would be long (approximately as in Received Pronunciation of *gate*); the second, *a*, would also be long (approximately as in RP *father*); *i* (as in *dip*) and *a* would be short; and the stress would fall on the second syllable. There is some degree of artificiality in this system, since *cepacia* is not a classical word but a later scientific coinage, formed from the classical Latin *caepa*. Indeed, this scholarly pronunciation does not correspond with any current pronunciations in the scientific and infectious disease communities. Any attempt to introduce it as a standard might paradoxically cause further confusion.

The standard pronunciation of Latin that scholars have reconstructed implies the primacy (for literary purposes) of the so-called Golden Age of Caesar, Cicero, and the Augustan poets and historians. Infectious disease specialists in the 21st century should not adopt this pronunciation, unless it is a genuinely useful and acceptable solution to a real problem. Our times, unlike the era of the Gileadites, do not deem mispronun-

ciation a capital offence. Classicists should be willing to help if they are asked but have no proprietary rights over the functional idiolect of modern scientific Latin whose users can use whatever pronunciation they find conducive to communication.

Dr. Moore, a member of the International *Burkholderia cepacia* Working Group, is principal clinical scientist in medical microbiology at the Northern Ireland Public Health Laboratory, Belfast City Hospital. His research interests include the development of molecular tools that characterize microbial pathogens in infectious diseases to aid in patient management, particularly the use of ribosomal RNA detection and sequencing techniques to detect etiologic agents of culture-negative infections.

Prof. Williams teaches of Greek at the Queen's University of Belfast. Much of his work has focused on Hellenistic poetry (especially that of Callimachus); he has also written on Babrius, Gregory of Nazianzus, Ovid, Juvenal, and Colluthus and has investigated linguistic usage, theophany, the relationship of the Cynics to early Christianity, polar bears in antiquity, and E.M. Forster's use of the Daphne story. He is now editing Cercidas of Megalopolis.

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Address for correspondence: John E. Moore, Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast, BT9 7AD, Northern Ireland; fax: +44 (28) 2589 2887; email: jemoore@niphl.dnet.co.uk

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# Travellers' Health: How to Stay Healthy Abroad, 4th edition

**Richard Dawood, editor**
**Oxford University Press, Oxford, U.K., 2002, ISBN: 0-19-262947-6, Pages: 762, Price: $19.95**

Since the first edition of this book was published in 1986, travel medicine has flourished as a specialty, with seemingly no end to the expansion of air travel and the number of intrepid persons seeking out remote destinations for work or pleasure. The original edition was immediately received as the best general guide available for health professionals who dispense advice and medications to travelers, as well as for travelers who want more information about potential hazards they might encounter. This fourth edition, which is even more comprehensive and authoritative than its predecessors, continues the original successful formula and can be unreservedly recommended to anyone, expert or non-expert, interested in the subject. Almost every conceivable topic is covered in the 75 chapters, each written by an expert or group of experts drawn mostly from the United Kingdom, with enough contributors from the United States to provide an international dimension.

One of the most useful sections, for travelers at higher risk, focuses on those who are pregnant, very young, elderly, and disabled, as well as those with HIV infection. Medical practitioners will find common topics addressed for patients with chronic, even life-threatening, conditions who ask about their fitness to travel and receive immunizations. Expedition health and medical kits are also well covered, and many chapters have references or guidance on accessing more information. A variety of sporting and recreational activities are discussed in individual chapters. Even humanitarian workers are considered with the inclusion of a chapter on land mines. Expatriates get a full discussion, including a warning not to habitually complain about their servants to expatriate colleagues, as well as a valuable section on personal security. Otherwise, the text follows the familiar disease and disease vector chapter format with excellent clarity and conciseness.

Acceptable online services supply up-to-date information for travelers on country-by-country health hazards, but none can compete with Dawood's guide. This gold mine of information is a fascinating read for even the armchair explorer. Travellers' Health is the authoritative guide for regular travelers, especially for anyone planning to live and work in foreign climes. For the health professional, the volume also serves as an accessible compendium of information about unfamiliar infectious diseases and environmental hazards and their prevention. And, despite the thoroughness of its content, the book has remained slim enough to pack away in the carry-on flight bag.

**Peter J. Baxter**

University of Cambridge, Cambridge, U.K.

Address for correspondence: Peter J. Baxter, University of Cambridge, Institute of Public Health, Robinson Way, Cambridge, CB2 2SR, United Kingdom; fax: 44 1223 336590; email: pjb21@medschl.cam.ac.uk

# Product Review: MicrobeCards

**MicrobeCards, Mark S. Pepler, 103 cards, $24.95, ISBN 1-5581-217-1, Washington, D.C., American Society for Microbiology Press, 2002.**

As someone whose professional life is split between teaching lay people to understand scientists and teaching scientists how to talk intelligibly to lay people, I am always looking for good teaching aids. Under the right conditions, MicrobeCards are definitely one such aid. MicrobeCards are a deck of 103 palm-sized (6 cm x 9 cm), color-coded flash cards, which collectively provide a surprisingly large amount of accurate, well-organized information about five categories of microbes: gram-positive bacteria, gram-negative bacteria, viruses, fungi, and parasitic organisms.

All cards use the same format. The front is color-coded by microbe category and provides full-color microscopy and clinical images. The back features a schematic of the human body, showing, at a glance, where the microbe causes disease. It also displays a standardized summary of five key features: pathogenesis, immunity, epidemiology, diagnosis, and control. The microbe's name appears on front and back, and all labeled illustrations on the front are keyed to points in the summary on the back.

The strengths of this product are its portability, comprehensiveness, and decidedly low-tech approach to teaching. Small enough to slip into a shoulder bag, MicrobeCards are crammed with a textbook's worth of information. Having trouble waking up during the morning commute? Use the next traffic jam to test yourself on *Streptococcus pyogenes*—is the photo on the left acute impetigo or necrotizing fasciitis? Do you want to strike up a conversation with someone in line at the Burger Doodle but can't think of a way to break the ice? Simply reach in your bag, hand him or her flash card 97, and have your new acquaintance test you on the pathogenesis of *Taenia saginata* (answer: "a) Encysted larvae are ingested in undercooked beef. Cysticerci are released, attach to the small intestine by b) a hookless head and grow unto adult worms up to 10 meters long in 3 months. c) Each segment of the worm (proglottid) has male and female sexual organs and is capable of producing over 1,000 eggs. Proglottids are motile and can migrate—for example, from the anus at night."). The card describes this experience as "disconcerting."

For science students who would rather hang out in Java Monkey than the library, these low-tech flash cards just can't be beat. In fact, marketing the product as MicrobeCards, not Microbe-PalmPilot, is a smart move. The detailed images, schematics, and linked text on MicrobeCards would be difficult to

encompass on a PDA, and laptops are really just too cumbersome to pull out and use in a traffic jam, while in line, or while sunning at the beach. Furthermore, this reviewer found the tactile learning experience (handling the cards, flipping each one back and forth, and watching the growing pile of "learned" cards) more satisfying than covering the same information by mousing from screen to screen.

Of course, all this convenience comes at a price. Most 20-year-old students may have no trouble reading the text on the back of these cards, but the angels-dancing-on-the-head-of-a-pin sized (33 characters per inch) had this reviewer renaming the pack MicroCards and wishing that ASM Press included a complimentary magnifying glass with every purchase.

Furthermore, as flashy as these cards are, a couple of small improvements would have made them more useful as, well, flash cards. Specifically, the microbe category color-coding, which appears only on the front of each card, should appear on the back to minimize the use of color as an accidental clue during memory and recognition drills. For the same reason, the name of each microbe should appear only on the back. I found ignoring the colored rims and covering each name with my thumb a bit awkward as I went through the deck, testing my memory (bad) and the cards' interest level (excellent).

The ideal users for these cards are undergraduate or graduate students (with really good eyesight) who are taking courses in medical microbiology or

infectious diseases. I wouldn't recommend the cards for high school use as they are written at a graduate reading level. On the other hand, life-long learners like my friend Jill, a self-taught polymath who seems to have equal rapport with both sides of her brain, may also find these cards exactly as advertised: A serious learning tool that's fun and friendly.

**Kimberly Sessions**

Emory Center for AIDS Research, Atlanta, GA

Address for correspondence: Kimberly Sessions, Emory Center for AIDS Research (CFAR), 1518 Clifton Rd. NE, Room 816, Atlanta, GA 30322, USA; fax: 404-727-9853; email: kbs@emory.edu



EMERGING INFECTIOUS DISEASES
A Peer-Reviewed Journal Tracking and Analyzing Disease Trends    Vol.8, No.5, May 2002

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## Conference Summary

# Converging Issues in Veterinary and Public Health

More than 20 key officials from the American Veterinary Medical Association and the Association of American Veterinary Medical Colleges met with staff from the Centers for Disease Control and Prevention (CDC) December 5–6, 2002, to discuss the increasing convergence of issues confronting human and animal health. Among the officials in attendance were the deans from more than half of all U.S. veterinary schools.

The meeting goals were to increase the veterinary community's understanding of CDC programs and the varied roles played by veterinarians throughout the agency; to provide CDC officials an opportunity to gain insight into current issues in veterinary medicine as well as the public health perspectives of veterinary leaders; and to provide a forum for discussions on ways to increase partnerships between the human and veterinary medical communities to meet critical public health needs. Presentations were made by James Hughes, Michel Bunning, Patricia Griffin, David Bell, Nina Marano, Tracee Treadwell, Thomas Ksiazek, and Peter Schantz, National Center for Infectious Diseases; Marguerite Pappaioanou, Office of Global Health; Hugh Mainzer and Andrew Dannenburg, National Center for Environmental Health; and Douglas Hamilton, Epidemiology Program Office. Many of these speakers are CDC veterinarians, who described their paths from veterinary training to public health.

The daily interactions of humans, animals, and the environment have a dramatic impact on public health. Current and evolving health threats include infections transmitted through animals, insects, food, and water, as well as illnesses resulting from environmental toxins, the misuse of antibiotics, and bioterrorism. Factors affecting these threats include the international movement of people, animals, and animal products; globalization and management of the complex food and fiber system; climate and other environmental changes, including those affecting wildlife populations and their interactions; and national and global security. Effectively meeting these challenges requires strong links between human and animal health clinicians, researchers, laboratorians, and public health officials.

Specific topics presented included West Nile virus and other vectorborne diseases, emerging viral and parasitic zoonoses, food safety, antimicrobial resistance, CDC's role in the 2001 anthrax investigations, and the agency's bioterrorism preparedness and response program. Presentations highlighted public health issues such as the need to upgrade containment facilities and to define optimal antibiotic use for farm animals. Efforts needed to further protect the health of humans, companion animals, zoo and exotic animals, and wildlife were also discussed. These efforts include improving strategies to reduce the occurrence of intestinal parasites in pets and increasing surveillance among imported animals and products to recognize infections not previously seen in the United States.

Several presenters emphasized the importance of surveillance systems in enabling prompt recognition of disease occurrences. Examples included two food safety surveillance programs: FoodNet, a collaborative project involving nine states, the U.S. Department of Agriculture, CDC, and the Food and Drug Administration; and PulseNet, a national and international network of public health laboratories that subtype foodborne bacteria to enable rapid comparison of DNA patterns through an electronic database. Other surveillance systems discussed included the National Antimicrobial Resistance Monitoring Systems and the Laboratory Response Network (LRN). LRN is a tiered system of laboratories with varying diagnostic capabilities, ranging from confirmatory analysis to specialized identification of agents potentially used in a bioterrorist attack. The network is supported through funding designated for bioterrorism preparedness and response. Meeting participants discussed the need to increase participation of veterinary clinicians and diagnosticians in these surveillance systems, especially LRN, noting that 80% of the agents classified as "category A" (i.e., those posing a major risk to national security because they can be easily disseminated or transmitted from person to person, result in high death rates, and require special efforts to ensure preparedness) are zoonotic. Strategies discussed at the conference toward this end included adding veterinary and animal health laboratories to LRN as well as establishing a similar network among such laboratories to collect more comprehensive data on the occurrence of infections affecting veterinary and human health.

CDC veterinarians participating in the meeting described their experiences as well as the roles of other agency veterinarians. Many CDC veterinarians are epidemiologists who joined the agency as officers in the Epidemic Intelligence Service (EIS), CDC's 2-year, hands-on comprehensive epidemiology and public health training program. Of the approximately 75 veterinarians who work at CDC, nearly half are in the National Center for Infectious Diseases, where they work in laboratory animal research as well as epidemiology. Discussions at the meeting described the critical roles played by veterinarians at the local, state, and national levels in responding to the recent West Nile virus outbreaks. Approximately 42 states currently have state public health veterinarians.

Many discussions focused on ways to increase the number of veterinarians in public health clinical and laboratory programs. Several CDC veterinarians cited classes in herd health as stimulating their interest toward public health careers. At the initial level, efforts are needed to ensure that veterinary students are aware of these career opportunities early in their education. Potential strategies include offering externships and public health rotations, such as at CDC or at local and state health departments, as

part of veterinary medical school training courses and offering combined degrees in veterinary medicine and public health (i.e., DVM/MPH)—a course of study already offered by several veterinary colleges. Other innovative public health programs that could be incorporated by veterinary medical colleges include studies in food safety, environmental toxicology, healthy ecosystems, international diseases, and population medicine.

More veterinary EIS Officers are also needed. Approximately one third of veterinarians applying to EIS are accepted, essentially the same acceptance rate as for other professions. Increased numbers of qualified veterinary applicants would therefore translate into higher numbers of accepted veterinarians. Similarly, efforts are needed to increase the number of veterinarians and veterinary students applying for other training programs at CDC such as the Emerging Infectious Diseases Laboratory Fellowships. Through this program, bachelor's or master's level scientists are recruited for

1-year assignments and postdoctoral level scientists for 2-year assignments at state, local, and CDC public health laboratories. More veterinary applicants are also needed for other training programs offered by CDC, such as the elective in epidemiology for senior medical and veterinary students—a 6- to 8-week introductory course in preventive medicine, public health, and applied epidemiology.

The World Health Organization (WHO) has recently published its findings from a study group on the future of veterinary public health (WHO Technical Report Series 907). The report describes the increasing emergence and reemergence of zoonotic diseases in the 1980s and 1990s and their importance for global public health (1).

To effectively meet these challenges, human and animal health issues must be merged into a new public health agenda. Creating and responding to such an agenda depends on strong interactions between the human and veterinary clinical, laboratory, and public health profes-

sional organizations. These interactions are essential for developing new and strengthening existing partnerships necessary for implementing effective public health programs. This meeting was a step toward this goal.

**Lonnie King\* and Rima Khabbaz†**

\*Michigan State University College of Veterinary Medicine, East Lansing, Michigan, USA; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

**Reference**

1. World Health Organization. Future trends in veterinary public health: report of a WHO study group. (Technical report series, No. 907). Geneva: The Organization; 2002.

Address for correspondence: Lonnie J. King, Dean, Michigan State University College of Veterinary Medicine, G100 Vet Med Center, East Lansing, Michigan, USA 48824-1314; fax: 517-432-1037; email: kinglonn@ cvm. msu.edu

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**Correction**

**Volume 8, Number 10**

In the letter to the editor, Evaluation and Validation of a Real-Time Polymerase Chain Reaction Assay for Rapid Identification of *Bacillus anthracis*, the author list should read as follows:

Alex R. Hoffmaster, Richard F. Meyer, Michael P. Bowen, Chung K. Marston, Robbin S. Weyant, Kathy Thurman, Sharon L. Messenger, Erin E. Minor, Jonas M. Winchell, Max V. Rassmussen, Bruce R. Newton, J. Todd Parker, William E. Morrill, Nancy McKinney, Gwen A. Barnett, James J. Sejvar, John A. Jernigan, Bradley A. Perkins, and Tanja Popovic.

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**Emerging Infectious Diseases**

**Policy on Corrections**

The Emerging Infectious Diseases journal wishes error-free articles. To that end, we

1. Make corrections as quickly as we become aware of errors
2. Publish corrections online and in print.

Online, we correct the error in the article it occurred with a note that the article was corrected and the date of correction. In print, we prominently publish a full correction, printing all needed information, and provide the URL of the corrected online article for reprints.

For additional information on corrections, send e-mail to eideditor@cdc.gov

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**EID**
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**Frank Day, Konkow Maidu (1902–1976).**
**The Water Test (c. 1970–1975).**
Oil on canvas, 48.26 cm x 62.86 cm.
Fine Art Collection, Heard Museum, Phoenix, Arizona, USA.

"Once in while I take up color and paint a little bit because if I do not do this, all things will be forgotten," Frank Day said of his work. The artist, who was born into the Konkow Maidu tribe in Berry Creek, California, was concerned that if not documented, his tribe's perception of the world would disappear (1). A gifted storyteller and teacher as well as talented artist, Day translated this tribal perception of the world into narrative images filled with Maidu themes in bold color.

Like many Native Americans of his generation, Day was under pressure throughout his life to abandon Native cultural practices in the interest of assimilation. A boarding-school student, he grew up wearing a standard school uniform and learning the ways of the broader society. But after the death of his father in 1922, Day set off to explore the history, language, ceremonies, and customs he had learned from him and other tribal elders. For a decade, he traveled western areas that had been inhabited by Indian tribes for hundreds of years and finally settled in California, where he worked as an agricultural laborer. After a serious injury, he turned to art as therapy. Without formal training, he soon exhibited untapped artistic talent and pure, distinctive style (1).

In the more than 200 canvasses he painted in the last two decades of his life, Day integrated myth, legend, and oral tradition into powerful compositions. His paintings, created from memory rather than observation, had a dreamy, symbolic, and imaginative bend. Rough brushstrokes, rich texture, and raw emotive color (2) invoked the spiritual underpinnings of cultural traditions rather than the traditions themselves. The paintings contained strong intuitive structure and contemporary elegance.

Day's "cultural memory" refuted presumptions that the California Indians were vanishing, and he was heralded for his inspiring presence during the revitalization of California Indian arts in the 1960s and 1970s. His artistic contributions were celebrated in "Memory and Imagination," a major exhibit organized by the Oakland Museum of California in 1997. Day's works are an authoritative tribute to Native American heritage and its focus on the spiritual connection between humanity and nature (3).

Infectious diseases (from smallpox and plague to tuberculosis and influenza) featured in many of the Indian legends whose essence Day sought to preserve (4). Blending the dangerous with the supernatural, these legends weaved historical accounts into tales of mystery, medicine, and magic and celebrated the creative spirit with which Native tribes approached disease survival. One painting, The Burning of the Roadhouse, commemorated therapeutic burning of dwellings to rid them of disease; another, Sunflower Remedy, portrayed a dazzling sunflower shielding a child from tuberculosis.

The Water Test, on this cover of Emerging Infectious Diseases, is a culmination of Day's Native Indian and artistic philosophy: everything is interconnected and imbued with spiritual energy that can be positive or negative. In this symbolic composition, human presence is in center stage. The pastoral scene, afloat in nature, is spare and horizontal but full of vitality. Water, a critical element providing not only physical but also spiritual sustenance, is set off by dramatic earth tones, balanced on the left by a thriving tree and in the diagonal center by a fallen one, which (as if charged by unknown energy) stretches to infinity. A man leans over the water, perhaps to test if it is clear enough to drink or warm enough to get into. Distracted by his reflection, he assumes a narcissistic posture and smiles at his robust image, his own character now being playfully tested by the water. His body is perfectly balanced and in control, but his relationship with the environment seems ambiguous. The water bank is teeming with oversized centipedes, some lurking in bellicose conference under a rock, some venturing out for prey. Their proximity, inflammatory colors, and poised poisoned fangs exude hostility.

The realistic encounter of man and water is embroidered with fantasy. The water contains invisible seeds of harm. The artist, acknowledging that the man's water test is as vain and elusive as his reflected image, pulls out of the water and into the foreground the centipedes, crude indicators of harm amplified and exposed to the naked eye. Our water tests are more refined, but they still seek indicators of harm. While we search for better evidence of their presence, harmful critters remain hidden. Standard plate counts or coliform counts are reasonable predictors of microbial presence, but as we peer deeply into our water, other microbes—noroviruses, Giardia, Cryptosporidium—continue to elude us, testing our essential drinking water and our survival.

**Polyxeni Potter**

1. Wilson DB. Frank Day: memories and imagination [cited 2003 February 7]. Available from: URL: http://maiduaction.org/htm/frank_day.htm
2. Walkingstick K, Marshall AE. So fine! Masterworks of fine art from the Heard Museum. Phoenix (AZ): Heard Museum; 2001.
3. Wasserman A. Memory and imagination: the legacy of Maidu Indian artist Frank Day [cited 2003 February 19]. Available from URL: http://www.museumca.org/exhibit/exhi_memory_imagination.html
4. McFarlan AA, editor. American Indian legends. New York: Heritage Press; 1968.

## Upcoming Infectious Disease Conferences

**April 2-5, 2003**
37th Annual Meeting of the European Society for Clinical Investigation
The Pathophysiology of Diseases: From Bench to Bedside
Verona, Italy
Contact: Giovanni Ricevuti, M.D.
phone: 39-0382-502499
Email: g.ricevuti@smatteo.pv.it
Website: www.esci.eu.com

**April 6-8, 2003**
Society for Healthcare Epidemiology of America (SHEA) Annual Meeting
Arlington, VA
Contact: SHEA Meetings Dept. (856) 423-7222 ext. 350
Website: www.shea-online.org

**May 1-3, 2003**
ISAAR 2003—4th International Symposium on Antimicrobial Agents and Resistance Antimicrobial Treatment in the 21st Century: Current Challenges and Future Strategies
Seoul, Korea
Contact: Ms. Susan Chung
Phone: 82-2-3410-0327
Fax: 82-2-3410-0023
Email: susan@ansorp.org
Website: http://www.ansorp.org/isaar2003/ intro.htm

**May 7, 2003**
International Society of Travel Medicine Conference
New York, New York
Contact: Brenda Bagwell
Phone: 770-736-7060
Fax: 770-736-6732
Website: http://www.istm.org

**May 7-11, 2003**
8th Conference of the International Society of Travel Medicine
New York City
Contact: Lisa Astorga,
Conference Manager

**May 10-13, 2003**
13th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Glasgow UK
Contact: Administrative Secretariat +41 61 686 77 11
Email: info@akm.ch
Website: www.escmid.org/eccmid20

# EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends        Vol.9, No.5, May, 2003

## Upcoming Issue

**For a complete list of articles included in the May issue,
and for articles published online ahead of print publication,
see http://www.cdc.gov/ncidod/eid/upcoming.htm**

### Look in the May issue for the following topics:

Planning against Biological Terrorism: Lessons from Outbreak Investigations

Vero Cytotoxin–Producing *Escherichia coli*
O157 Gastroenteritis in Farm Visitors,  North Wales

Pandemic Influenza and Healthcare Demand in
the Netherlands: Scenario Analysis

Human Milk Secretory Antibodies against Attaching
and Effacing *Escherichia coli* Antigens

*Aeromonas* spp. as a cause of traveler's diarrhea:
Clinical features and antimicrobial  resistance

The Global Burden of Rotavirus Disease in Children

*Entamoeba moshkovskii* Infections in Children

Eliminating Trachoma in Areas with Limited Disease

Discovery of Chronic Wasting Disease in Free-Ranging Wisconsin White-Tailed Deer

Puumala Virus Infection with Acute Disseminated Encephalomyelitis and Multiorgan

Probiotics and Prebiotics: Where Are We Going?"

## 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.

# EMERGING INFECTIOUS DISEASES

**EID Online** www.cdc.gov/eid

**Influenza** (p.304)

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