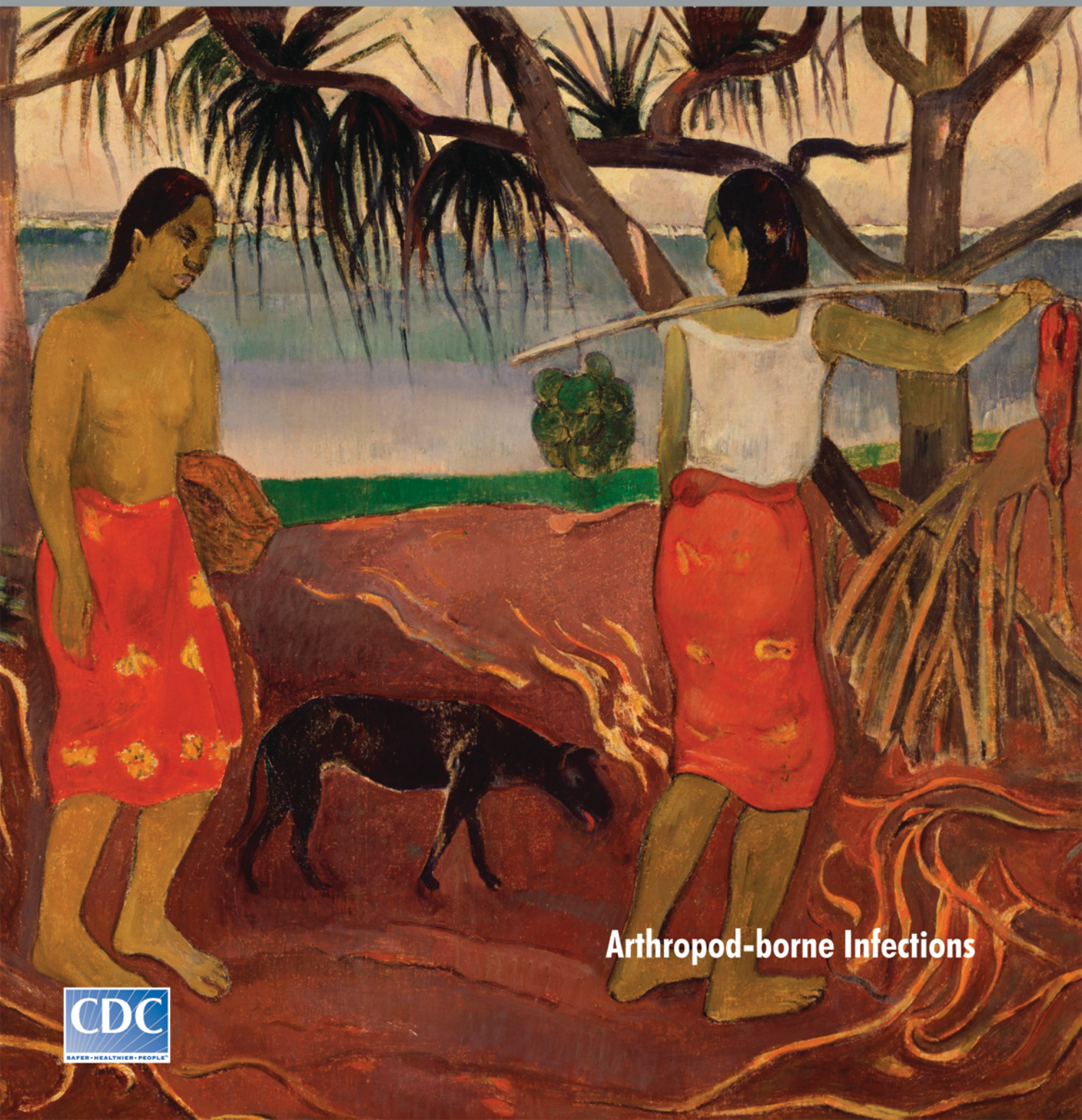


# EMERGING INFECTIOUS DISEASES

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Vol.10, No.4, April 2004



Arthropod-borne Infections





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Paul Gauguin (1848–1903). *I Raro te Oviri (Under the Pandanus)* (1891)  
Oil on canvas (73.03 cm x 91.44 cm).

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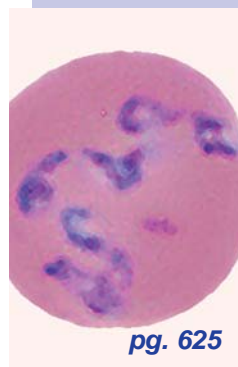
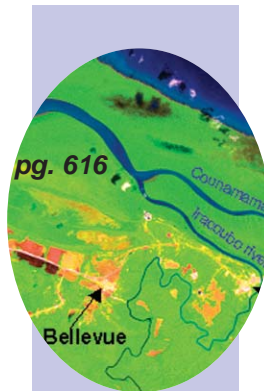
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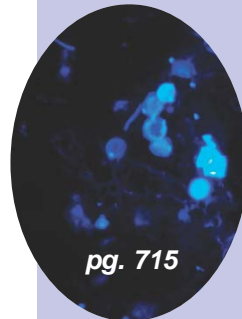
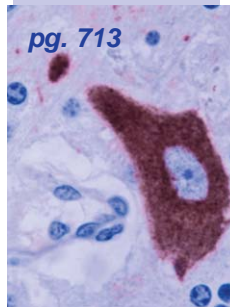
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# Economics and Preventing Hospital-acquired Infection

Nicholas Graves\*

The economics of preventing hospital-acquired infections is most often described in general terms. The underlying concepts and mechanisms are rarely made explicit but should be understood for research and policy-making. We define the key economic concepts and specify an illustrative model that uses hypothetical data to identify how two related questions might be addressed: 1) how much should be invested for infection control, and 2) what are the most appropriate infection-control programs? We aim to make explicit the economics of preventing hospital-acquired infections.

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Approximately 1 in 10 hospitalized patients will acquire an infection after admission, which results in substantial economic cost (1). The primary cost is that patients with hospital-acquired infections have their stay prolonged, during which time they occupy scarce bed-days and require additional diagnostic and therapeutic interventions (2). Estimates of the cost of these infections, in 2002 prices, suggest that the annual economic costs are \$6.7 billion per year in the United States (3)<sup>1</sup> and £1.06 billion (approximately US \$1.7 billion) in the United Kingdom (4).

The economic rationale for preventing hospital-acquired infections has been discussed (5,6) and can be summarized as follows: hospital-acquired infections take up scarce health sector resources by prolonging patients' hospital stay; effective infection-control strategies release these resources for alternative uses. If these resources have a value in an alternative use, then the infection control programs can be credited with generating cost savings; these infection control programs are costly themselves, so the expense of infection control should be compared to the savings.

For many hospital infections, the costs of prevention are likely to be lower than the value of the resources released (4,7,8), even when costs "are estimated liberally and the benefits presented conservatively" (9). Under these circumstances, infection control should be pursued, since more

stands to be gained than lost (5). We attempt to make explicit the concepts on which these arguments rely and, in particular, concentrate on providing a framework for answering two questions: how much in total should we invest in prevention for any given infection-control situation, and how should this investment be allocated among competing infection-control strategies? Our aim is to make the economics of prevention explicit while using a minimum of technical language, algebra, and economics jargon.

## Concepts and Definitions

### Valuing Resources Attributable to Hospital-acquired Infection

Infection uses hospital resources. By preventing infection, these resources are saved. For some of these resources, the associated expenditures may be terminated, and the savings would be expressed in terms of cash-savings, for example saving on drugs, consumables, and nursing staff employed on a contract that can be terminated at short notice. However, expenditures associated with many resources are difficult to avoid in the short term, and conserved resources cannot be easily, or costlessly, exchanged for cash. A longer-term obligation to the resource may exist due to a contractual commitment, such as an employment contract with a staff member or a lease agreement for a diagnostic device, or a physical commitment, such as investment in buildings, capital equipment, and infrastructure.

These differences illustrate the differences between fixed and variable costs. While cash-savings from avoided variable costs are easy to quantify, the resources that represent fixed costs cannot be exchanged for cash in the short-term. Researchers have found that 84% (10) and 89% (4) of the costs of hospital care are fixed in the short term. Furthermore, expenditures made to acquire fixed resources, recorded by cost-accountants, may or may not

---

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<sup>1</sup>Care should be taken in interpreting this estimate, as it was derived from data gathered in the mid-1970s (Study on the Efficacy of Nosocomial Infection Control–SENIC).

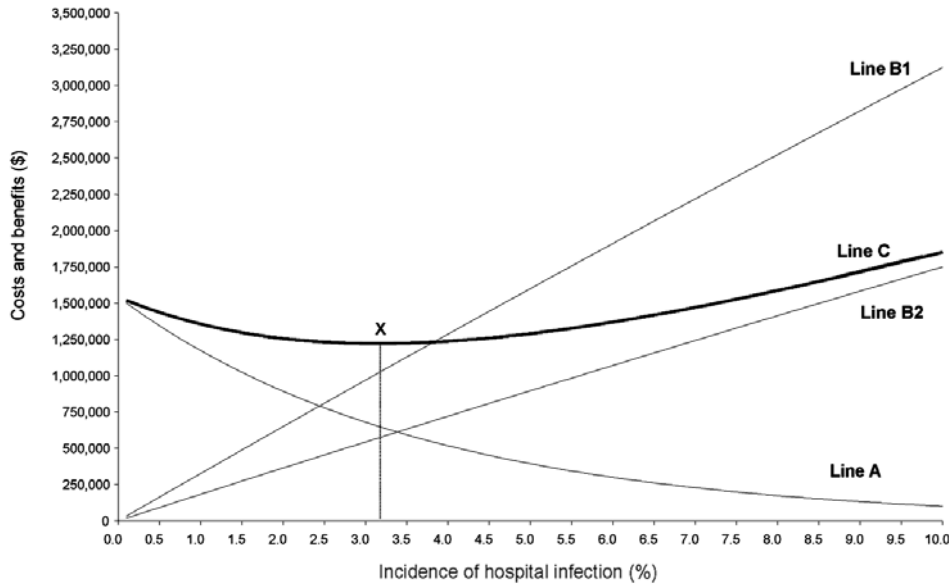


Figure 1. A model of investment in infection-control activities. Line A, cost and effectiveness of infection control; line B1, gross costs of infection and benefits of prevention; line B2, net costs of infection and benefits of infection control; line C, total costs; point X, incidence that minimizes total costs.

be an accurate assessment of their economic value. Because financial expenditures on fixed costs are unavoidable in the short-term, they are largely irrelevant to decision-making in the short-term. For economic analysis, we prefer to explore the value of the best alternative use of the resources that are fixed in the cost structure of the hospital. This value is the opportunity cost of the resource.

### Perspective for Economic Evaluation

Many have argued that the benefits of infection control are widespread. Treating infection represents an economic burden to the hospital, and prevention saves these costs (4,11–20); however, less is known about other benefits. One reason might be that hospital administrators, who hold the purse strings for infection control, are primarily interested in savings to their budgets and do not focus on other benefits that might arise for patients, informal caregivers, or other healthcare agencies (20). A broader perspective might include the monetary value of avoided illness and death from hospital infection. Attributing excess illness and death to hospital infection, however, is difficult, and accurately valuing these very real costs is fraught with problems. Still, when a narrow perspective is adopted, and costs and benefits other than those that fall directly on the hospital sector are excluded, economic analyses may underestimate the social benefits of infection-control programs.

### Incremental and Marginal Analyses

Incremental and marginal analyses are concerned with changes to “cost” and “benefit” in respect to the status quo (existing hospital expenditures and their outcomes) (21). If the existing budget for infection control is \$100,000 and a new infection-control program costs \$40,000, the total cost

of infection control will increase to \$140,000. The incremental cost of the new program is the change in total cost from \$100,000 to \$140,000, or \$40,000. If implementing this program avoids 50 bloodstream infections, then the incremental benefits are 50 avoided infections. Marginal analysis is similar but refers to a change of just one unit, say \$1 or one infection. Most infection-control programs would cause incremental changes, not pure marginal changes.

### Infection-Control Investment and Strategies

In the sections that follow, we adopt the perspective of a hospital administrator and only examine costs and savings to the hospital. We do not seek to determine a social value of the health benefits of avoiding hospital-acquired infection, so the estimate of the benefits of infection control is conservative. We also assume that all decisions are made within the short term; this is the time frame in which fixed costs cannot be changed. The model illustrated in Figure 1 uses hypothetical data to analyze the costs and benefits of prevention and provides answers to both questions: 1) how much to invest for infection control and 2) which are the most appropriate infection-control programs.

### How Much To Invest for Infection Control

The horizontal axis in Figure 1 represents an incidence of wound infections in 50,000 patients undergoing hip replacement. The vertical axis represents cost and potential savings. Line A summarizes the relationship between the cost and the effectiveness of infection control strategies. To achieve the low incidence of 0.01% requires an investment of resources in infection control valued at \$1.5 million. However, to reduce rates to only 5.00% requires a lesser investment of \$393,661. Line B1 represents the



gross costs of hospital infection, i.e., the gross savings that would result from prevention. These costs and potential savings increase with incidence. The primary cost of hospital infection is the loss of bed-days due to prolonged length of stay. Care must be taken in valuing these bed-days and other resources used for hospital infection (22). For economic analysis, consider what else could be done with the resources released by prevention. A hospital in which rates of infection are successfully reduced will have more bed-days available, so new patients can be admitted. The value of these new admissions to the hospital represents the gross costs of infection and, therefore, the potential gross savings from prevention. For example, if demand for hip replacement is such that patients, their insurers, or the public medical system is prepared to pay \$1,250 to the hospital for each additional case treated, then the opportunity cost of wound infection is the revenue that could be earned by treating extra cases with the bed-days used by hospital infection. In Appendix 1 (available online at: <http://www.cdc.gov/eid/vol10no4/02-0754.htm#app1>), we illustrate how to calculate these costs for an incidence of 10.00% and 5.00%, and these data are used to plot line B1 in Figure 1.

So far we have restricted our discussion of the cost and savings from prevention to changes in the use of bed-days. We should also consider the financial expenditures made by the hospital. The financial expenditures on resources that represent fixed costs are largely irrelevant, as they cannot be avoided in the short-term. However, fixed costs are certainly being used more productively.<sup>2</sup> More relevant are the variable or discretionary costs that change in response to a decrease in the incidence of hospital infection. First, patients who previously would have stayed for 15 days with a hospital infection now stay only 10 and will incur lower variable costs.<sup>3</sup> If the decrease in variable costs from reducing length of stay by 5 days is \$100 per patient, then line B1 in Figure 1 is too low an estimate of the costs of infection and the potential savings from prevention. However, variable costs will also increase as a result of the increase in patient turnover. At rates of zero infection, hospitals are treating 2,500 more patients than before, and this will cause an increase in variable costs. For example, the capacity to perform the surgery will have to be increased, requiring more surgeons, anesthetists, operating room nurses, and prostheses and other consumables. If the increase in variable cost is evaluated at \$750 per new admission, then this must be offset against the \$100 per patient reduction in variable costs and the \$1,250 increase in revenue per case. The result is the net costs of infection and net savings from prevention. In Appendix 2 (available online at: <http://www.cdc.gov/eid/vol10no4/02-754.htm#app2>), we illustrate how to calculate these costs for an incidence of

10.00% and 5.00%. This suggests that the gross cost of infection (the gross savings from prevention), marked by line B1, is incorrect. We indicate the correct values, the net cost of infection (the net savings from prevention), by line B2.

Line C in Figure 1 is the total cost to the healthcare system and is the sum of lines A and B2 for every incidence rate of hospital infection. For example, at an incidence of 9.00%, the net cost of infection is \$1,582,536 (Line B2), and the cost of prevention programs is \$132,088 (Line A). The sum of these at an incidence of 9.00% is \$1,714,624 (Line C).

The incidence of infection that minimizes total cost, indicated by Line C, is marked with an X in Figure 1, and achieving this incidence represents a rational objective for policy makers. To explore this point further, consult Appendix 3 (available online at: <http://www.cdc.gov/eid/vol10no4/02-0754.htm#app3>), which includes the values used to plot lines A, B2, and C between the incidence rates of 2.9% and 3.4%. We conclude that point X is a rational policy goal because, at this point, marginal savings exactly compensate the marginal investments in prevention. In contrast, investments that drive infection rates lower than point X are not adequately compensated. The data included in Appendix 3 show that the last infection we should prevent will cost \$17,810 in terms of infection-control activities and will release resources worth \$17,810.

The investment in prevention that achieves the rate indicated by point X is therefore the correct budget constraint for infection control. At point X, there is no net gain or loss, which signals the best achievable, or equilibrium, outcome.

### Determining Appropriate Infection-Control Programs

There are many different ways of preventing hospital infections and therefore many different ways of moving toward point X. Choices have to be made among the numerous competing infection-control programs available. To help make these choices, we apply the technique of incremental cost-effectiveness analysis (23), where the costs of the interventions are represented in monetary terms, and the benefits are measured in natural units common to all interventions under consideration. For this example, the benefits of the infection-control programs are

<sup>2</sup>At rates of 10% the fixed costs of the organization were used to treat 50,000 patients, but at zero rates of infection, 52,500 patients were treated with the same volume of fixed costs; this represents an improvement in efficiency. See Appendix 1, available online at <http://www.cdc.gov/ncidod/EID/vol10no4/02-0754.htm#app1>

<sup>3</sup>Reductions might be in expenditures on antimicrobials to treat the infection, the equipment used to deliver therapy, and on resources used for wound care such as dressings, irrigations, and other consumables. Also, the workload of the nursing staff may be reduced, so expenditures on agency nurses might be reduced.

Table. Cost, effectiveness, and benefits of six competing infection-control strategies

Option	Incremental cost of prevention	Incremental benefit <sup>a</sup>	Effect <sup>b</sup>
Option 6	\$299,611	1,942	4.00%
Option 3	\$523,487	1,205	2.50%
Option 2	\$643,487	3,346	6.80%
Option 5	\$812,457	3,448	7.10%
Option 1	\$874,512	1,059	2.20%
Option 4	\$892,931	3,960	8.00%

<sup>a</sup>Cases prevented.

<sup>b</sup>Reduction in incidence.

the number of cases of infection avoided. We should choose the infection-control programs that minimize the cost per infection avoided while remaining within the budget constraint identified by point X.

A useful first step is to identify a patient group and an infection to prevent. Keeping with the example of infection in hip replacement, the next step is to identify all reasonable strategies that might prevent this type of infection. In our example, we propose six strategies and assume that all available prevention strategies are represented by these six options. The cost, effectiveness, and benefits of each are illustrated in the Table, and these data are plotted in Figure 2. Options 1 to 6 compete with each other, and only the most appropriate will be used.

The status quo is an incidence of 10.00% for a population of 50,000 patients who receive a new hip in a given period. Option 6 is clearly preferable to options 1 to 5 because the cost of preventing one infection by this mode is only \$154, calculated by dividing the cost of option 6 by the benefit of option 6, both relative to the status quo. This is an incremental cost-effectiveness ratio (ICER). See Appendix 4 (available online at: <http://www.cdc.gov/eid/vol10no4/02-0754.htm#app4>) to clarify how to calculate

ICERs. In our example, the hospital should first invest \$299,611, moving from the origin to option 6.

Now, all other options (except option 6) are still available, and any further decisions must be evaluated with respect to option 6, the new status quo. Both option 1 and option 3 are less effective and more costly than the status quo (option 6) and so are excluded. Option 2 beats options 4 and 5; although all prevent further infections, option 2 does so at the lowest cost. The hospital should invest a further \$343,876, moving from option 6 to option 2. The status quo is now option 2, and only options 4 and 5 remain, with the final move being to option 4.

The question of which are the most appropriate infection-control programs has been answered. A policy represented by a line that joins the origin to the points marked option 6, option 2, and option 4 illustrates the most appropriate, most cost-effective, infection-control strategy.

We have pursued the most cost-effective pathway without considering point X, where total costs to the healthcare system are minimized. Consider the information included in Figure 3. This is a version of Figure 1 that includes the incremental costs and benefits of the six competing strategies described above. The status quo, at an incidence of 10%, and the moves to options 6, 2, and 4 that define the cost-effective pathway are marked. The figure shows that the hospital should not invest beyond the point defined by option 2. While a further move to option 4 is the lowest cost alternative for preventing further cases of infection, option 4 exceeds the budget constraint and ultimately increases costs to the healthcare system (line C).

**Discussion**

Many have considered the economics of preventing hospital-acquired infection. We argue, with the exception

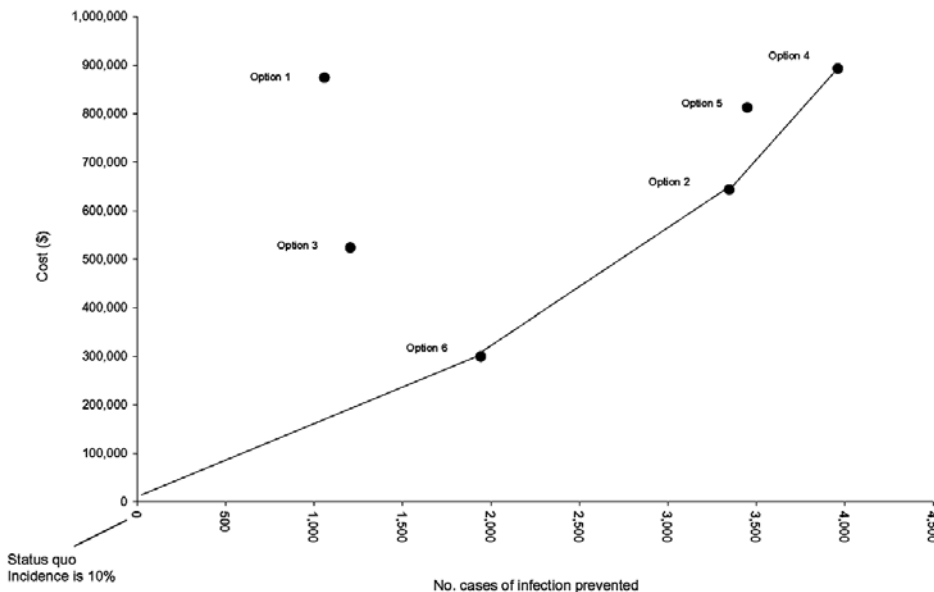


Figure 2. A model of cost, effectiveness, and benefits of six competing infection-control strategies.



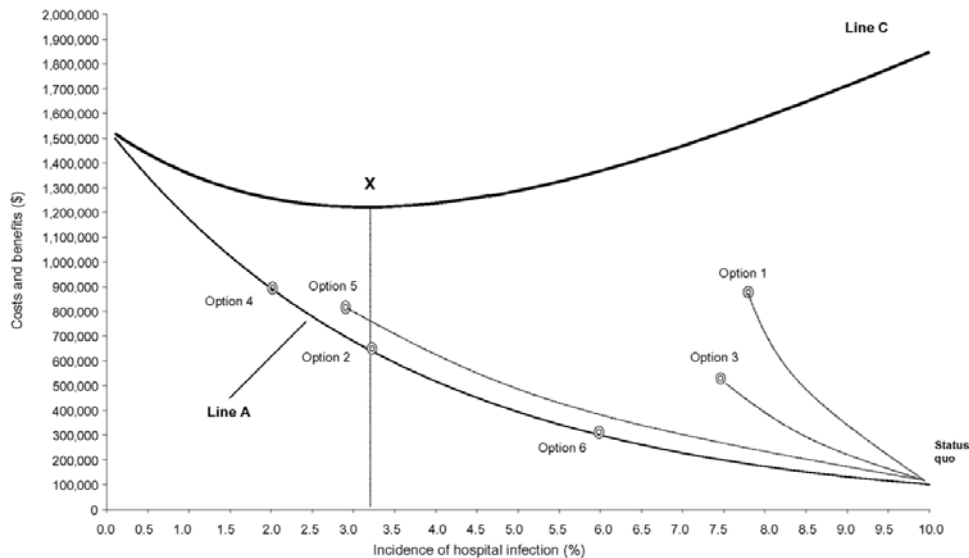


Figure 3. Six competing infection-control strategies imposed on the model of investment in infection-control activities.

of one study (24), the complexity of the economic issues has been neglected. In this article we attempt to make the economics explicit. We demonstrated how the concept of opportunity cost might be used to value the costs of hospital infection and therefore the savings from infection control programs. We argue that existing literature uses financial costs to represent the cost of infection, and this method may lead to erroneous conclusions. Financial costs are a monetized estimate value of health-services cost (25) and might not satisfy the definition of opportunity cost. We offer an explicit treatment of how variable costs change in response to infection control and highlight the difference between the gross and net costs of hospital infection. We also suggest that, as the perspective for the analysis broadens, the costs of infection and the potential benefits of infection control increase. This will affect the position of point X in our example and, therefore, affect infection control policy. Finally, we identify a budget constraint for infection control where the costs of prevention are compensated by simultaneous cost-savings and illustrate how incremental cost-effectiveness analysis might be used to identify the most efficient choices for infection control.

To build the model we propose requires data to plot lines B2 and A; obtaining these data will allow line C to be estimated and point X to be identified for any given hospital infection scenario. Plotting line B2 requires data on the incidence of hospital infection and the resulting opportunity costs. Although a complicated task, progress is being made with the specification of models (26,27), and establishing the true effect of hospital infection on length of stay and cost is now a more rigorous process. Deriving values of alternative uses of these bed-days represents further challenges. Due to the absence of a reliable market mechanism for health care, finding an accurate valuation for a marginal admission to a hospital is difficult

(28), as is finding the opportunity cost of bed-days. Further research in this area is required. Plotting line A requires that the cost and effectiveness of competing infection control strategies be understood. Although the number of economic evaluations that include an assessment of costs and benefits of infection-control strategies are limited (29), a broad and diverse literature exists on the effectiveness of many infection-control interventions. The quality of the evidence is likely to be variable, encompassing a range between correctly designed, randomized controlled trials and subjective, expert opinion. If the findings could be synthesized in a rigorous manner, uncertainty characterized, and summary estimates of the likely effectiveness derived, the costs of these strategies could be estimated separately and the data required to plot line A procured. With data to plot lines A and B2, line C, and point X can be estimated. Achieving this for the numerous patient groups and sites of hospital infection will be a major task, but the conceptual framework, expertise, and data are available for an explicit treatment of the economics of preventing hospital infection.

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# Reanalyzing the 1900–1920 Sleeping Sickness Epidemic in Uganda

E.M. Fèvre,\* P.G. Coleman,† S.C. Welburn,\* and I. Maudlin\*

Sleeping sickness has long been a major public health problem in Uganda. From 1900 to 1920, more than 250,000 people died in an epidemic that affected the southern part of the country, particularly the Busoga region. The epidemic has traditionally been ascribed to *Trypanosoma brucei gambiense*, a parasite now confined to central and western Africa. The Busoga region still reports sleeping sickness, although it is caused by *T.b. rhodesiense*, commonly believed to have spread to Uganda from Zambia in the 1940s. Our analysis of clinical data recorded in the early 1900s shows that the clinical course of sleeping sickness cases during the 1900–1920 epidemic in Uganda was markedly different from *T.b. gambiense* cases, but similar to *T.b. rhodesiense*. These findings suggest that *T.b. rhodesiense* was present in Uganda and contributed to the epidemic. The historic context is reassessed in the light of these data.

Uganda is affected by Gambian sleeping sickness, which is caused by infection with *Trypanosoma brucei gambiense*, and Rhodesian sleeping sickness, which is caused by *T.b. rhodesiense*. *T.b. rhodesiense* occurs in the eastern part of the country, whereas *T.b. gambiense* occurs in the northwestern part of the country. From 1900 to 1920, the Busoga region of Uganda experienced a large-scale epidemic of the disease, during which an estimated 250,000 people (1), a third of the population of the region, died (Figure 1). It is believed that the species of parasite responsible for this first documented epidemic in Uganda was *T.b. gambiense* and that *T.b. rhodesiense* was introduced there in the 1940s when another, smaller epidemic was identified in the same region. However, this idea has been the subject of some debate (2,3).

The first published description of sleeping sickness cases in the 1900–1920 epidemic was made by A.R. and J.H. Cook in their Church Missionary Society (CMS)

Hospital at Mengo on February 11, 1901 (4,5). The extent of the epidemic became clear as the number of case-patients seen at that hospital increased and as the disease was identified around the northern shore of Lake Victoria (6,7). The study of sleeping sickness at the time focused on discovering the causative agent; being newly recognized in Uganda, the disease had not been previously described in eastern Africa. The discovery of trypanosomes as a disease agent and their mode of transmission was relatively recent. Bruce (8) described transmission of cattle trypanosomes by tsetse flies in Zululand in 1895; the causative agent of “Trypanosoma fever” in the Gambia had been indicated in 1902 by Forde (9) and described by Dutton (10) as *Trypanosoma gambiense* (now classified as *T.b. gambiense*). At the time of the Ugandan outbreak, Manson (11) believed that the disease was linked to *Filaria perstans* (now known as *Mansonella perstans*), a blood-dwelling nematode of no clinical importance. Early efforts in Ugandan patients infected with sleeping sickness focused on detection of *F. perstans*. *T.b. rhodesiense* was described for the first time (in present day Zambia) in 1910 (12), and *T.b. rhodesiense* was confirmed in Uganda during the next major epidemic 30 years later (13).

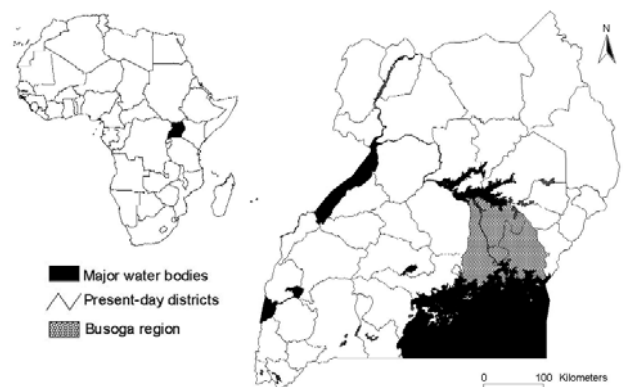


Figure 1. Map of Uganda showing its location in East Africa (inset) and the location of the Busoga region where approximately 250,000 people died from 1900 to 1920.

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## A New Disease?

It is not known how long sleeping sickness may have existed in Uganda before 1900. Most of the pioneering scientists assumed it was a new problem to the region because they found no evidence that it had occurred there before, and they assumed that the disease always occurred as an epidemic. However, Christy (7) noted that sleeping sickness had probably been present long before it was first documented and that it probably originated in Busoga, the core of the present day *T.b. rhodesiense* focus. Similarly, on reviewing the available evidence, Duke (14) states that “some form of human trypanosomiasis” had occurred around the Ugandan shores of Lake Victoria prior to the epidemic.

Confusion over the existence of sleeping sickness in this area was intertwined with the available knowledge of the causative organism. We have already seen that *T.b. gambiense* was the only human-infective trypanosome to have been described at the time. Furthermore, sleeping sickness was recognized in many parts of central Africa (15). It had not been documented previously in eastern Africa, and the assumption was that the Ugandan disease was an extension of the epidemic raging westward in the (present day) Democratic Republic of Congo.

When the epidemic began in Uganda, Castellani (16) noticed two groups of distinct clinical symptoms among the patients. The infection in the first group he called Trypanosoma fever, as it was similar to the disease seen in the Gambia and ascribed to *T. gambiense* (10,17). The second infection he called sleeping sickness, and tentatively called the trypanosome that he found in those cases *T. ugandense* (16). The distinction was essentially clinical; what he called sleeping sickness was a much more virulent infection than the Trypanosoma fever caused by *T. gambiense*. Bruce et al. (18) described two cases of a disease “not unlike Trypanosoma fever” in two patients who had recently come to Uganda (whom he termed Nubians). The patients, a policeman and a prisoner, had arrived from the present day Sudan, where *T.b. gambiense* occurs today. Bruce et al. (18) later insisted that the less acute “Trypanosoma fever” symptoms were simply the first stage of full-blown sleeping sickness caused by *T. gambiense*.

If the sleeping sickness had been due to *T.b. gambiense*, it would suggest that the parasite was imported from the west as part of large-scale human population movements that occurred at the time (15). Only Köerner et al. (2) have questioned the identity of the parasite responsible for the first Ugandan epidemic. They argue that as *T.b. rhodesiense* has occurred in stable endemic foci that can expand and cause epidemics, *T.b. rhodesiense* was probably present in Busoga long before 1901. This is an attractive argument, as the wholesale replacement of one parasite species by another (*T.b. gambiense* by *T.b. rhodesiense*) in a region

seems unlikely, and such a replacement has not been recorded in any other sleeping sickness focus. In addition, *T.b. rhodesiense* is primarily a zoonotic parasite in which human-to-human transmission is thought to occur rarely, and human movements (15,19) in isolation from movements of the zoonotic reservoir (20) may not be sufficient to account for its introduction.

## Hypothesis

Here we test the hypothesis proposed by Köerner et al. (2) that the parasite responsible for sleeping sickness in the Busoga and surrounding regions of Uganda from 1900 was *T.b. rhodesiense*. This organism causes an acute disease, resulting in death after a period of 3–12 months (21,22), while *T.b. gambiense* causes a chronic infection, with which a person may go about daily activities for many months or years (21,23), despite occasional and often mild symptoms. Our analysis, therefore, is based on comparison of the survival time of sleeping sickness patients estimated from clinical notes recorded during the Ugandan epidemic, with survival times of known *T. b. rhodesiense* patients from the current disease focus in southeast Uganda, and *T. b. gambiense* patients in western and central Africa.

## Methods

### Archives

The Mengo Hospital archives (CMS Mission Hospital at Mengo), which include original patient case notes made by the Cook brothers, are held in the archives section of the Mulago Hospital at the Makerere Medical School in Kampala, Uganda. The first sleeping sickness patient recognized in Mengo was admitted on February 11, 1901 (Figure 2). The geographic distribution of the cases seen at this hospital extended across a wide area, although most of the patients came from the close vicinity. Many patients reporting in the latter years of the epidemic were referred to the specialist hospital run by the Royal Society Sleeping Sickness Commission in Entebbe (set up as part of the Commission’s study on the disease), or later to the sleeping sickness isolation camps on islands in Lake Victoria. Some details of the treatments prescribed and numbers of cases seen in these camps are available (24,25). At this time in the development of therapy for sleeping sickness, no effective drugs were available for the disease, so death of the patients was due to sleeping sickness and, rarely, treatment side-effects; the data included here show the true clinical course of the disease in untreated patients.

### Data Collection

For each patient treated at the Mengo Hospital with diagnosed sleeping sickness, full details as they appeared in the archives were entered into a database, which was

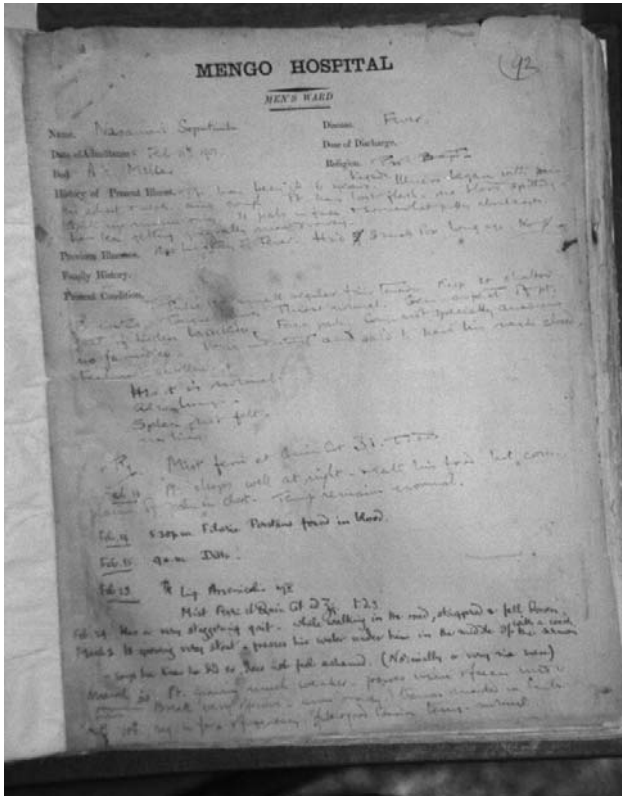


Figure 2. Case notes of the first recorded sleeping sickness patient in the Mengo Hospital case records.

used for later analyses. This electronic database has been made available to the archivists in Mengo. Full details were entered for patients seen through 1910; beyond this time, many patients were turned away. In addition, the authorities were managing to bring the epidemic under control, and the number of cases was diminishing. Additional data were also extracted from the Reports of the Royal Society Sleeping Sickness Commission (6,18).

### ***T.b. rhodesiense* Comparison**

Odiit et al. (22) published data on duration of symptoms in 30 patients who died of sleeping sickness within a week of seeking treatment for *T.b. rhodesiense* infection at the Livestock Research Institute sleeping sickness hospital in Tororo, Uganda, between 1988 and 1990.

### ***T.b. gambiense* Comparison**

Adams et al. (26) conducted postmortem examinations on 16 patients with fatal *T.b. gambiense* infections and state that few fatal cases are documented and that duration of illness before death is “rarely established”; this accounts for the scarcity of time-to-death data in contemporary literature. One source of *T.b. gambiense* clinical data (27) presents important details of the clinical course of the disease to death but could not be used in this analysis as no

estimates were made of the length of illness before treatment or death. However, Yorke (28) does present a summary of untreated cases between 1908 and 1919 in various countries in West Africa. These countries include the French Congo (present-day Republic of Congo), the Gambia, and the Belgian Congo (present-day Democratic Republic of the Congo). Full details for each patient are not provided, and the survival times for the different groups of patients were taken as the average of the range given (e.g., five patients who were followed-up after 2–3 years were each given a survival time of 2.5 years). These data were supplemented by several more recently published case histories for *T.b. gambiense* patients who were treated in the United States and Europe after various periods of travel in Africa (23,29–33); the date of the last visit to Africa before diagnosis was taken as the infection date for these patients, and survival time was taken to the point of first treatment or death. Cases of congenital sleeping sickness from this literature were excluded.

### **Statistical Analyses**

A survival analysis (34) was conducted to compare the time from onset of symptoms to death of the Ugandan patients in the Mengo archives and Sleeping Sickness Commission Reports (6,18) from 1901 to 1910, and known *T.b. rhodesiense* (22) and *T.b. gambiense* patients (23,28–33). The criteria for including a patient from the Mengo archive dataset were that the case had been recorded as a sleeping sickness death following inpatient stay in the hospital, that the length of the hospital stay was recorded, and that the length of time of illness before admission had been recorded in the clinical notes. That is, an estimate of the total time of the clinical course of the illness, from onset of symptoms to death, was available. The same criteria were applied to the clinical notes appearing in the Sleeping Sickness Commission Reports (6,18). The Kaplan-Meier survival analyses were conducted in *S+* 2000 (MathSoft, Inc., Cambridge, MA) and the survival curves were compared using the log-rank (Mantel-Cox) test.

### **Results**

#### **Descriptive Statistics**

From 1901 to 1910, a total of 11,767 case-patients were recorded in the Mengo inpatient records. This figure excludes all patients admitted in 1902 because all the records from that year were destroyed in a fire. Just over 1% of these (204 cases) were sleeping sickness cases. The outcome of admission was biased in favor of discharge (160 cases). Five patients were referred to the Royal Society sleeping sickness hospital, and 24 deaths were recorded. Of these 24 deaths, 11 (Table 1) untreated



Table 1. Time sick pre-admission and total time to death for 11 patients admitted to Mengo Hospital, 1901–1910

No.	Sex	Approx. age (y)	Months sick pre-admission	Admission date	Date of death	Time in hospital (mo)	Time sick predeath (mo)
2	F	11	3	Feb 13, 1901	Mar 8, 1901	0.77	3.77
3	M	30	1	Apr 20, 1901	May 8, 1901	0.60	1.60
4	F	10	6	Apr 25, 1901	May 8, 1901	0.43	6.43
13	M	-	3	Jul 29, 1901	Aug 28, 1901	1.00	4.00
18	M	-	3	Sep 16, 1901	Oct 19, 1901	1.10	4.10
26	M	18	0.03	Jun 28, 1903	Jul 4, 1903	0.20	0.23
133	M	20	0.20	Jun 23, 1907	Jul 8, 1907	0.50	0.70
136	M	40	24	Jul 3, 1907	Jul 10, 1907	0.23	24.23
171	F	40	1	Apr 24, 1909	May 4, 1909	0.33	1.33
181	M	20	5	Mar 1, 1910	Mar 12, 1910	0.37	5.37
189	M	45	1	Jun 9, 1910	Jun 24, 1910	0.50	1.50

patients had complete records of date of admission and death and of the duration of symptoms before admission. Sixteen cases from the Sleeping Sickness Commission Reports were included with these (Table 2). All 30 cases presented by Odiit et al. (22) were included, and 88 untreated, diagnosed case-patients were extracted from Yorke (28). Of these, 54 had died and 34 were still alive on follow-up (accounted for by censoring in the survival analysis). Eight contemporary *T.b. gambiense* patients were included (23,29–33), all of whom survived to treatment.

### Survival Curves

The Kaplan-Meier Survivorship curves resulting from the analysis of these data are shown in Figure 3. The median survival times were 2 months, 4 months, and 36 months for the Tororo 1988–1990, Mengo Hospital Plus Sleeping Sickness Commission reports 1901–1910, and western African datasets, respectively.

### Log-Rank Test

The log-rank test showed no significant difference between the Ugandan 1901–1910 and 1988–1990 survival

rates ( $\chi^2 = 1.7$ ;  $df = 1$ ,  $p = 0.12$ ). Therefore, the clinical course from onset of symptoms to death in this sample of patients from the 1901–1910 epidemic in southern Uganda was not significantly different to that of patients in Tororo with *T.b. rhodesiense* infections from 1988 to 1990. The Ugandan 1901–1910 and West African survival rates were significantly different ( $\chi^2 = 184$ ;  $df = 1$ ,  $p < 0.001$ ) and the Ugandan 1988–1990 and West African survival rates were also significantly different ( $\chi^2 = 175$ ;  $df = 1$ ,  $p < 0.001$ ). The clinical course of the disease during the Ugandan 1901–1910 and 1988–1990 epidemics is significantly shorter than the clinical course of sleeping sickness experienced by the West African *T.b. gambiense* patients.

### Discussion

The clinical course of sleeping sickness during the period from 1901 to 1910 Ugandan epidemic does not differ from contemporary *T.b. rhodesiense* cases in Uganda (22). The duration of illness from first onset of symptoms of documented *T.b. gambiense* patients did differ significantly from both the Ugandan 1901–1910 and Ugandan 1988–1990 patients. The clinical course from the

Table 2. Time sick preadmission and total time to death for 16 patients admitted to the Royal Society Sleeping Sickness Commission Hospital before 1903

Ref.	Patient no.	Months sick preadmission	Time in hospital (mo)	Time sick pre-death (mo)
(6)	35	4	4.5	8.5
(6)	23	1	2.1	3.1
(6)	18	3	1.4	4.4
(6)	40	2	1.2	3.2
(6)	25	7	1.2	8.2
(6)	22	2	1.1	3.1
(6)	41	3	1.4	4.4
(6)	37	2	1.9	3.9
(6)	38	6	1.7	7.7
(6)	15	1	1.8	2.8
(6)	36	2	0.4	2.4
(6)	42	3	1.9	4.9
(18)	28	8	7.0	15.0
(18)	20	6	3.6	9.6
(18)	21	3	2.5	5.5
(18)	79	1	1.6	2.6

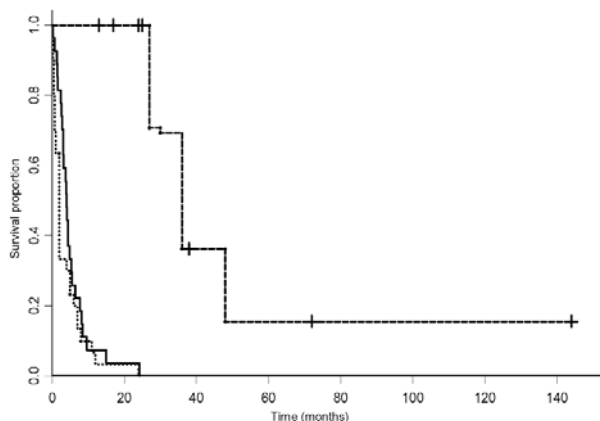


Figure 3. Results of the survival analysis (confidence intervals not shown), showing the proportion of patients surviving over the period from first onset of symptoms for the different groups of patients. The dashed line is the West African dataset from Yorke (28) (vertical bars represent censored observations). The solid line is the 1901–1910 Mengo and Sleeping Sickness Commission datasets and the dotted line the Tororo 1988–1990 dataset from Odiit et al. (22).

1901–1910 epidemic is consistent with infection with parasites belonging to the *T.b. rhodesiense* subspecies and is consistent with the hypothesis proposed by Köerner et al. (2). Unfortunately, no archived parasite material exists for this period, which would allow the molecular confirmation of this result by screening for the *SRA* gene (35), which is specific to *T.b. rhodesiense*, or for screening with *T.b. gambiense*-specific molecular markers (36).

Given these findings, how might the observations made by Castellani (16), that two distinct clinical pictures were sometimes observed in Uganda at the time, be explained? The designation as “Nubians” of some of the patients suggests that these patients were from Sudan. They were certainly migrants and may well have been carrying *T.b. gambiense* parasites before migrating that were discovered on examination in Uganda. Although local transmission of these parasites cannot be excluded, *T.b. gambiense* was probably not responsible for the widespread deaths in the Busoga region generally. Most of the cases described by Christy (7) as he roamed around Uganda were of an acute disease. Hodges, who was the Medical Officer for the Uganda Protectorate, states that the time to death of Ugandan patients from realization they were sick was 3–4 months (24), based on a great many observations, and that the duration of illness rarely exceeded 10 months. This observation is further echoed by Low and Castellani (37), who state that “very chronic cases, running a course of more than a year’s duration, are very rare.” *T.b. gambiense*, if it did exist concurrently, would probably have been limited to those areas where migrant workers for the British authorities were allowed to settle. These settlement areas were purposefully estab-

lished away from tsetse-infested bush in the efforts to control the epidemic (38).

If both species of human infective trypanosome (*T.b. gambiense* and *T.b. rhodesiense*) were in Uganda during the 1901–1910 epidemic, and both were treated at the Mengo and Sleeping Sickness Commission hospitals, the data acquired from the archives for deaths would be biased towards clinical descriptions of *T.b. rhodesiense*. The *T.b. gambiense* data presented here demonstrate that many of the patients are treated before death. For similar reasons, *T.b. gambiense* would be poorly represented among deaths in Mengo and the Sleeping Sickness Commission; patients would have been discharged, as the symptoms would not have been considered serious enough for them to remain in the scarce hospital beds. Also, mixed infections with both *T.b. rhodesiense* and *T.b. gambiense* could have occurred if *T.b. gambiense* was being transmitted locally; however, due to the acute nature of *T.b. rhodesiense* infections, patients infected with both parasites would most likely have been seen with *T.b. rhodesiense*-like symptoms.

Therefore, the results of this analysis cannot exclude *T.b. gambiense* as a cause of illness among some patients but can positively include *T.b. rhodesiense* as a cause of sleeping sickness at the time. The epidemic is likely to have been due to *T.b. rhodesiense* with occasional cases of *T.b. gambiense* in patients who had migrated from *T.b. gambiense* foci on the present northwestern border of Uganda (e.g., “Nubians”). Case number 136 from Mengo (Table 1) may well be one of the occasional *T.b. gambiense* case-patients (or the result of recall errors), as the patient reported having been ill for 24 months. This one case does not affect the outcome of the analysis, however (Figure 3).

### Rinderpest and Cattle Restocking

If, as these data suggest, the 1900–1920 epidemic was due, at least in part, to *T.b. rhodesiense*, the question arises as to its causes, and the cause of the spread of sleeping sickness through the whole of the region to previously unaffected areas. There is no doubt that the onset of the colonial administration in Uganda resulted in social changes and population mobility (39), which had important environmental consequences (39). In referring to the causation of *T.b. gambiense* epidemics in central Africa, Lyons (19) blames the disruptive effects of colonization. Human movements had, however, occurred regularly throughout Africa’s history (2), and the situation in Uganda was doubtless more complex.

Some other trigger, in conjunction with these factors, likely was involved in spreading sleeping sickness from the endemic foci outwards. It has been suggested that this might have been rinderpest, an infectious disease of livestock (39). The start of the sleeping sickness epidemic

coincides with the end of the rinderpest pandemic in cattle (39,40). In the early 1890s, and for the decade that followed, rinderpest, or cattle plague, ravaged most of Africa. Millions of cattle died from this virulent viral infection (40), causing sociologic and ecologic upheavals throughout the continent. Although rinderpest is often linked to the sleeping sickness outbreaks in eastern Africa, it has been possibly linked for the wrong reasons. The disease-induced cattle depopulation is generally thought to have resulted in a change in the dominant vegetation in the whole region; pasture lands reverted to bush and the distribution of tsetse-fly vector of sleeping sickness expanded (41). Although these ecologic changes occurred, they may not have been directly responsible for the spread of sleeping sickness.

The movement of cattle during livestock restocking (20) may be linked to the introduction of *T.b. rhodesiense* sleeping sickness, a zoonosis with a principally domestic cattle reservoir, to previously unaffected areas that resulted in serious outbreaks of disease. With the large-scale local and regional movements of animals that occurred after the rinderpest pandemic, as animals were traded in the cattle-depopulated areas, trypanosomes may have moved with them. In conjunction with the expansion of tsetse distributions as the ecology changed, the setting was ripe for a major sleeping sickness problem. Ford (39) notes that in setting up the Uganda Protectorate, a great deal of cattle movements occurred, either as groups moved away from areas under British control, or in search of post-rinderpest pasture. He also points out that the culture of large-scale cattle trading was well established. Local cattle movements as part of the restocking would have been extensive. *T.b. rhodesiense* could have spread from the core of the Busoga and other endemic foci to other tsetse-infested areas all around the northern Lake Victoria shore.

Recent molecular studies also challenge the conventional wisdom that *T.b. rhodesiense* spread through East Africa and to Uganda from Zambia (13), where it was first described. Tilley et al. (42) and Hide et al. (43) suggest that *T.b. rhodesiense* retains a stable genetic constitution through time and show that strains from Zambia are phylogenetically quite distinct from *T.b. rhodesiense* in Uganda. It is therefore unlikely that the parasite spread from there to Uganda. Rather, as Köerner et al. (2) suggested, *T.b. rhodesiense* has probably been present in southeast Uganda, either at endemic or epidemic levels (or both at different times), for hundreds of years. The dynamics of the spread of the disease, involving cattle movements and restocking (20), have probably been similar since the first association of cattle and humans in tsetse-infested areas of this part of the continent.

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# Pediatric Influenza Prevention and Control

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Global evaluation of influenza vaccination in children indicates that current recommendations are not followed. Most children at high risk for influenza-related complications do not receive the vaccine, and increased efforts are needed to protect them. Furthermore, vaccinating healthy infants 6–23 months of age and their close contacts should be strongly encouraged. Vaccinations are recommended for children with recurrent acute otitis media or recurrent respiratory tract infections and possibly for healthy daycare and school-age children because of the potential socioeconomic implications of influenza. Issues that need to be addressed include educating physicians and parents concerning influenza-related illness and complications, cost-effectiveness and safety of licensed vaccines, adequate vaccine supply, and availability of intranasal products.

Influenza vaccination is routinely recommended in pediatric patients of age  $\geq 6$  months who are at high risk for influenza-related complications because they have an underlying disease or are undergoing long-term aspirin therapy and are at risk of developing Reye syndrome (1–4). Administering the vaccine to healthy children is recommended only when they live with persons at high risk (1–4), although the Advisory Committee on Immunization Practices is gradually moving toward a recommendation to vaccinate all children ages 6–23 months (because of their substantially increased risk for influenza-related hospitalizations) and children ages 2–18 years who are household contacts of children ages  $0 \leq 23$  months (1–7).

Although health authorities in industrialized countries agree with these guidelines, use of influenza vaccine in clinical practice differs. Most children at high risk for complications do not receive the vaccine, and its use in healthy infants is not routinely accepted (8–10), even though results of recent studies suggest expanding the number of children for whom vaccination should be recommended (11–14). We discuss current vaccination practices in children, reasons and possible remedies for low immunization rates, and the possibility of extending its use in pediatrics.

## Vaccine Practices for Children

High-risk children for whom influenza vaccination is routinely recommended include those with chronic disorders of the cardiovascular or pulmonary system (including asthma), chronic metabolic diseases (including diabetes mellitus), chronic renal dysfunction, and hemoglobinopathies or immunosuppression (including cases caused by medications or by HIV) (1–4). Although an association between these conditions and an increased risk for influenza complications was first suggested many years ago (15,16), the level of vaccination in such children is still much lower than recommended, although it is slightly higher when children are followed up in specialized centers rather than by primary care physicians (perhaps because children seen in specialty clinics have more severe underlying illnesses), or when data regarding immunization are collected after implementing a reminder and recall system (8–10,17–19). One study of health maintenance organizations reported influenza vaccination rates of 9% to 10% among children with asthma and a rate of 25% among those attending an allergy and immunology clinic (17). The use of a reminder and recall system increased vaccination coverage among children with asthma from 5% to 32% (18). The highest coverage was found among pediatric patients attending a cystic fibrosis treatment center, in whom a vaccination level of 79% was reached (19). Data collected in Italy confirm that the behavior of pediatricians is not in line with the official recommendations. Among the 274 high-risk children attending the University of Milan's Pediatric Emergency Department during winter 2002–2003, the vaccination level was 26.3%; the highest rates were in children with HIV infection (52.3%), and the lowest rates were in those with asthma (9.5%) (10).

Few data concern the effect of encouraging vaccination in healthy children  $< 2$  years. However, comparing immunization rates among children of this age without any high-risk condition attending the University of Milan's Pediatric Emergency Department during the two winter seasons of 2001 to 2002 and 2002 to 2003 (after the publication of the suggestion that healthy children  $< 2$

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years be vaccinated) showed only a marginal increase (2.4% vs. 3.6%) (10).

### Reasons for Low Immunization Rates and Possible Solutions

Seven main obstacles to complying with recommendations for vaccination in children exist: 1) lack of understanding of the risk for influenza complications in children; 2) lack of knowledge of annual immunization's efficacy in primary prevention; 3) parents' negative reaction to parenteral vaccine administration ("Not another shot!"); 4) need for two priming doses in children <9 years old followed by annual administration; 5) fear of limited protection in younger and high-risk children; 6) concerns about possible adverse events; and 7) lack of precision in current recommendations. The most important of these obstacles are lack of understanding of the risks for complications and lack of knowledge of efficacy (10,20).

A number of studies of adult (particularly elderly) populations have shown that knowing risk factors for influenza complications, favorable perceptions of the vaccine, and clinician recommendations are the main variables predicting the administration of influenza vaccination (1,21,22). However, pediatric data indicate that some providers do not recognize influenza's clinical relevance, even when it occurs in children with severe underlying disease (8,9). A study designed to ascertain the self-reported use of influenza vaccine among pediatric oncologists found that approximately 30% did not think that influenza infection is important in children with cancer (8) and consequently do not recommend immunization. The central role of physicians' opinions in determining vaccination coverage is supported by data collected in a cross-sectional study of a group of children hospitalized during the influenza season in the United States (9): >70% of the children were vaccinated if a physician had recommended it to their parents, but 3% were vaccinated if no such recommendation had been made. A lack of awareness that children can receive influenza vaccine was a commonly cited reason for nonvaccination (9).

The attitude of pediatricians towards influenza vaccine can be explained by the fact that its importance in high-risk children and healthy infants is mainly suggested by indirect data. Although a number of studies have shown that influenza can significantly increase hospitalization, outpatient visits, and drug consumption in high-risk children of all ages (15,16), few trials (mainly involving children with asthma) have demonstrated that vaccination is clinically useful in reducing influenza-related complications (23,24). Furthermore, data concerning the efficacy of influenza vaccine in healthy infants <2 years of age have been collected from small groups. Although a reduction in influenza-like illnesses has been shown, the data do not evaluate

the importance of vaccination in reducing hospitalizations or complications (25,26). Pediatricians may be definitively convinced of the importance of preventing influenza and personally start supporting the use of vaccine when more data are available demonstrating its efficacy in children. Consequently, studies evaluating the real clinical impact of influenza vaccine, not only in children with risk factors but also in healthy infants, are needed.

Another probable factor preventing the use of influenza vaccines in pediatrics is that those currently licensed for use in children are parenteral (two injections for children <9 years of age being vaccinated for the first time) and require annual administration to maintain protection (1,3,27). Parents may be concerned about the number of injections their children receive during the course of routine early child health visits. Given the large number of vaccinations already included in the routine childhood immunization schedule, the addition of another "shot" may not sound attractive to parents and certainly not to their children. However, the availability of intranasal influenza vaccines may substantially reduce this problem (28). Recent advances in influenza vaccination include the development of a trivalent, cold-adapted, live-attenuated, intranasal vaccine that appears to be as effective as its intramuscular counterparts and induces a good immune response (including local immunoglobulin [Ig] A responses and secretory IgA antibodies that can protect against pathogens infecting mucosal sites) (29). One of the disadvantages of this vaccine is that individual susceptibility to infection with live viruses (and consequent immunogenicity) varies widely; vaccine strains' reversion to their wild-type genotype has also been considered a potential risk, although there is no evidence that this occurs (29). If eventually licensed for use worldwide, intranasal vaccines can be expected to increase influenza vaccination coverage, especially in children.

Concerns that influenza vaccine may offer limited protection and fears of possible adverse events are further reasons for its limited use in pediatrics (20). However, protective antibody levels after influenza vaccination have developed in 70% to 90% of children as young as 6 months of age, although fewer younger infants seroconvert, and some high-risk children may have a lower antibody response (1). Childhood vaccination programs fail to be beneficial if vaccine efficacy falls to  $\leq 25\%$ , levels that have never been reported in younger or high-risk children (1). Moreover, although mild local and systemic reactions to the vaccine may occur more frequently in persons who have never been exposed to the viral antigens it contains (e.g., young children), the currently licensed parenteral vaccines are generally safe and well-tolerated (1). Considering the possible effect that "vaccine-adverse" parents have on immunization policy in some regions, dis-



seminating information concerning the safety, tolerability, and immunogenicity of influenza vaccination in healthy infants and high-risk children is important.

Influenza prevention recommendations imprecisely describe the characteristics of high-risk children, contributing to inadequate vaccination in this population. For example, the Advisory Committee on Immunization Practices recommends yearly influenza vaccination for immunosuppressed children, including those with immunosuppression due to medications (1) but does not specify which diseases require vaccination, the doses of the immunosuppressive drugs, or the timing of the vaccination in relation to their administration (1). Conversely, the American Academy of Pediatrics states that the optimal time to immunize these children is when their peripheral leukocyte count is  $>1,000/\mu\text{L}$  and that vaccination has to be deferred during high-dose corticosteroid administration (27). These discrepancies reflect a lack of data and may explain why pediatricians have different approaches in clinical practice. Specific and uniform guidelines for each group of children at high risk would be the best way to overcome this problem. Still, in many clinical scenarios decisions are based on the best information available, and recommendations cannot deal with each and every situation that the medical provider confronts.

Globally evaluating the main reasons for low influenza vaccination coverage in pediatrics suggests that improving knowledge of influenza among pediatricians and parents could improve vaccination practices. The medical community spends substantial amounts of time with parents trying to convince them of the need for routine vaccinations, but in many instances, vaccines are suggested on the basis of the parents' or the healthcare providers' perception of vaccine or diseases of greatest importance. If parents lack insurance, economic considerations also become an issue. A change of mindset is needed to enhance acceptance of influenza vaccination; providing materials to educate parents would help effect this change. As television and print advertising promotes other pharmaceutical products, similar advertising could effectively promote influenza vaccination. The first step is to define simple, unequivocal, and practical guidelines specific to different groups of children at high risk and healthy infants  $<2$  years of age. These guidelines, for distribution to hospital physicians and primary care pediatricians, would contain detailed information regarding the consequences of influenza in such children and describe the effectiveness of influenza vaccine and the risk for adverse events. In addition, pediatricians can use recall systems to provide timely reminders for all patients.

### **Influenza Vaccine in Children Not at Risk**

In addition to the children for whom influenza vaccine

is already recommended or strongly encouraged, other pediatric patients can receive clinical benefits from its use. One group of children who could be included on the list of vaccination candidates is those with recurrent episodes of acute otitis media (AOM). Recurrent AOM is common in infants and children, and its possible sequelae make prevention desirable (30). Until a few years ago, chemoprophylaxis and controlling environmental risk factors were considered the best ways to reduce the incidence of new episodes of AOM in otitis-prone children, but the emergence of drug-resistant bacteria after antimicrobial drug administration raises questions about the advisability of drug therapy (13,30). Immunoprophylaxis against respiratory viruses has received growing attention because viral infections (including influenza) are associated with many, if not most, episodes of AOM. Data showing that administering parenteral, inactivated influenza vaccine can decrease the incidence of AOM by approximately one third strongly support the use of vaccination in preventing AOM (31). The demonstration that live-attenuated, cold-adapted, intranasal vaccine causes a 30% reduction in the incidence of febrile AOM in healthy children without a history of ear disease leads to the same conclusion (25). The importance of influenza vaccination in children with recurrent AOM has been recently demonstrated by Marchisio et al., who used an intranasal, inactivated, virosomal subunit vaccine (13). In this study, 133 children aged 1–5 years with recurrent AOM (defined as  $\geq 3$  episodes in the preceding 6 months or  $\geq 4$  episodes in the preceding 12 months) were randomized to receive the vaccine ( $n = 67$ ) or no vaccination ( $n = 66$ ). During a 6-month period, 24 vaccine recipients (35.8%) experienced 32 episodes of AOM, and 42 control participants (63.6%) experienced 64 episodes. The overall efficacy of vaccination in preventing AOM was 43.7% (95% confidence interval 18.6 to 61.1,  $p = 0.002$ ) (Table 1) (13). Moreover, the cumulative duration of middle ear effusion was significantly less in the vaccinated children (58.0% vs. 74.5%;  $p < 0.0001$ ) (13). As reducing the occurrence of AOM in children with recurrent episodes can have substantial clinical and socioeconomic effects, these data suggest that influenza vaccine can be considered a valid option in preventing the disease in otitis-prone children.

A second group of children who could be considered for influenza vaccine are those with recurrent episodes of respiratory tract infections (RRTIs). A large number of children without any immunologic problems experience multiple episodes of RRTIs during the first years of life; although these generally have a benign prognosis, they can cause substantial medical and socioeconomic problems (32). They are mainly caused by viruses and, during epidemic periods, influenza viruses can also be causative. Data collected in a recent study indicate that vaccinating

Table 1. Effectiveness of influenza vaccine as indicated by the occurrence of febrile respiratory illness and acute otitis media (AOM), and the use of antibiotics in children during the 6 months after vaccine administration<sup>a</sup>

Variable	Vaccine recipients, n = 67 (%)	Control participants, n = 66 (%)	Vaccine efficacy, %	p value
Febrile respiratory illness	55 (82.1)	63 (95.5)	13.2	0.03
≥1 course of antibiotics	26 (38.8)	42 (63.6)	38.9	0.007
≥1 AOM episode	24 (35.8)	42 (63.6)	43.7	0.002
≥2 AOM episodes	6 (9.0)	16 (24.2)	63.1	0.03

<sup>a</sup>Modified from P. Marchisio et al. (13).

children with RRTIs against influenza is effective in decreasing respiratory-related illness among them and their families (14). A total of 127 children 6 months to 9 years of age with a history of RRTIs (≥6 episodes per year if ≥3 years; ≥8 episodes per year if <3 years) were randomized to receive the intranasal virosomal influenza vaccine (n = 64 with 176 household contacts) or a control placebo (n = 63 with 173 household contacts). During the influenza season, vaccinated children had fewer respiratory infections or febrile respiratory illnesses, received fewer prescribed antimicrobial and antipyretic drugs, and missed fewer school days than the controls (Table 2); similar benefits and a reduced loss of parental work were observed among their household contacts (14). These results show that the benefits of influenza vaccination extend to children with RRTIs and their families and suggest that its use in such children should be encouraged.

### Influenza Vaccine in Healthy Daycare and School-Aged Children

A number of studies have shown that otherwise healthy daycare and school-aged children are most frequently affected by influenza, and high attack rates can substantially diminish their quality of life and disrupt everyday activities (33–36). Children shed larger quantities of influenza viruses for longer periods of time than adults and thus play an important role in spreading infection in their families and communities (1,37). Negative effects of influenza in otherwise-healthy children can extend to unvaccinated household contacts, who may require substantial diagnostic and therapeutic interventions and miss a number of school or working days. Neuzil et al. found that, during the

influenza season, the number of household members who became ill within 3 days of a child's absence from school was 2.2 times higher than expected. Excess absenteeism from work also occurred among parents (34). In line with these observations, we have found that the household contacts of children with influenza require more medical visits, miss more working or school days, and need more help at home to care for ill children than the household contacts of children without influenza (36).

Preventing influenza by vaccination can improve these situations. A blinded, placebo-controlled study of two influenza vaccines (an inactivated split-virus vaccine and a live-attenuated, cold-adapted vaccine) in 555 school-aged children in Russia demonstrated that both were efficacious in preventing school absenteeism by reducing the number of missed school days by 47% to 56% compared to missed school days in unvaccinated children (38). Similarly, in a study of the effect of an inactivated, split-virus vaccine on healthy children attending daycare or school in Italy during the years 2001–2002, we found that the vaccinated children experienced fewer upper and lower respiratory tract infections, received fewer antimicrobial and antipyretic prescriptions, and missed fewer school days because of respiratory illnesses (39). These data suggest that the effect of influenza on otherwise-healthy daycare or school-age children may be more substantial than is usually thought, encouraging wider pediatric use of influenza vaccine to reduce the overall extent of infection.

Strong support for wider pediatric use comes from evaluating the household impact of influenza vaccination in healthy daycare and school-age children. In a 1995 randomized, controlled trial of influenza vaccine for preschool

Table 2. Respiratory illness among children with recurrent respiratory tract infections and effectiveness of the influenza vaccine during the follow-up period<sup>a</sup>

Event	Vaccinated children, n = 64 <sup>b</sup>	Controls, n = 63 <sup>b</sup>	Vaccine effectiveness, % <sup>c</sup>	p value
No. of upper respiratory tract infections	2.95 ± 1.33 (3)	4.06 ± 2.13 (4)	27	<0.0001
No. of lower respiratory tract infections	0.67 ± 0.88 (0)	1.01 ± 1.12 (1)	33	0.03
No. of febrile respiratory illnesses	1.60 ± 1.39 (1)	2.06 ± 2.14 (2)	23	0.02
No. of hospitalizations	0.05 ± 0.10 (0)	0.10 ± 0.25 (0)	60	0.34
No. of antimicrobial prescriptions	1.31 ± 1.33 (1)	2.35 ± 1.59 (2)	44	<0.0001
No. of antipyretic prescriptions	2.16 ± 2.03 (2)	3.98 ± 2.37 (4)	45	<0.0001
Missed school days	5.35 ± 8.14 (6)	13.83 ± 12.50 (10)	61	<0.0001

<sup>a</sup>Modified from S. Esposito et al. (14).

<sup>b</sup>Mean values ± standard deviation (median in parentheses).

<sup>c</sup>Vaccine effectiveness: 1 minus attack rate (defined as rate of illness divided by total population) among vaccinated children divided by attack rate among controls.

children, the rate of febrile respiratory illnesses was 42% less among the unvaccinated household contacts of influenza-vaccinated children than among those living with unvaccinated children (40). Moreover, data collected in Tecumseh, Michigan (41), and Japan (7) indicate that mass vaccination of school-age children correlates with a reduced rate of respiratory illness and all-cause community death rate, which suggests that larger scale immunization can affect community epidemics. Similarly, during the 2001–2002 influenza season in Italy, we found that, compared to the household contacts of unvaccinated children, family members of influenza-vaccinated healthy children experienced fewer respiratory tract infections, needed fewer medical visits, missed fewer working days, and required less help at home to care for ill children (Table 3) (39). All of these findings highlight the fact that influenza in otherwise-healthy children attending daycare centers or schools has a considerable effect on their families and that the benefits of influenza vaccination extend to the family members of vaccinated persons.

The socioeconomic importance of influenza in childhood is confirmed by economic analyses showing that vaccinating healthy preschool and school-age children can lead to health and economic benefits during epidemic and pandemic periods (42–45). These studies used different analytic methods, outcomes, and costs but came to a common conclusion: vaccinating healthy children against influenza leads to a net cost saving, and the greatest financial benefit is observed when the vaccine is administered in a group setting (42–45). Savings are primarily due to avoided indirect costs and, in particular, reduced parental absenteeism from work.

## Conclusion

Global evaluation of the effect of influenza in pediatric patients indicates that influenza vaccination should be more widely used than is usually recommended. To protect them against the complications of influenza, increased efforts are needed to identify and recall high-risk children. Further, immunizing infants 6–23 months of age and their

close contacts is recommended. Children with recurrent AOM or a history of RRTIs and healthy children attending daycare centers or schools should also be included among the pediatric groups recommended for vaccination.

These conclusions are based on clinical and socioeconomic considerations arising from evaluating the impact of influenza vaccination on both the children themselves and their household contacts. Improved recognition of the complications of influenza in the first years of life, with resources dedicated to provider and public education on this issue, can help reduce obstacles to using influenza vaccine. Parents might choose vaccination for their children if they were more informed about the health and economic cost of influenza, its annual attack rate in childhood (which leads to days lost from school and work), and the central role of children in disseminating the infection in households and communities. The issues that need to be addressed include educating physicians and parents about the illness caused by influenza, the cost-effectiveness and safety of licensed vaccines, adequate vaccine supplies, and the availability of intranasal products. Improved compliance associated with nasal administration should increase the use of influenza vaccination. Only a heightened and regular demand for influenza vaccine will result in sufficient vaccine supplies at all times (not just on a year-to-year basis) and place us in a better position to detect a novel pandemic influenza virus strain.

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Table 3. Effectiveness of influenza vaccine among household contacts of children receiving influenza vaccine and unvaccinated controls<sup>a</sup>

Event	Household contacts of vaccinated children (n = 728) <sup>b</sup>	Household contacts of unvaccinated controls (n = 370) <sup>b</sup>	Vaccine effectiveness, % <sup>c</sup>	p value
No. of respiratory tract infections	3.03 ± 1.68	4.27 ± 1.68	30	0.0005
No. of medical visits because of respiratory illness	2.18 ± 1.37	3.16 ± 1.77	32	0.002
Loss of maternal work, days	3.22 ± 1.86	4.78 ± 2.34	33	0.001
Loss of paternal work, days	0.56 ± 0.46	0.98 ± 2.24	43	0.001
Help at home to care for ill children, days	0.57 ± 0.37	3.22 ± 2.24	83	<0.0001

<sup>a</sup>Modified from S. Esposito et al. (39).

<sup>b</sup>Mean values ± standard deviation.

<sup>c</sup>Vaccine effectiveness: 1 minus attack rate (defined as rate of illness divided by the total population) among household contacts of vaccinated children divided by attack rate among household contacts of controls.



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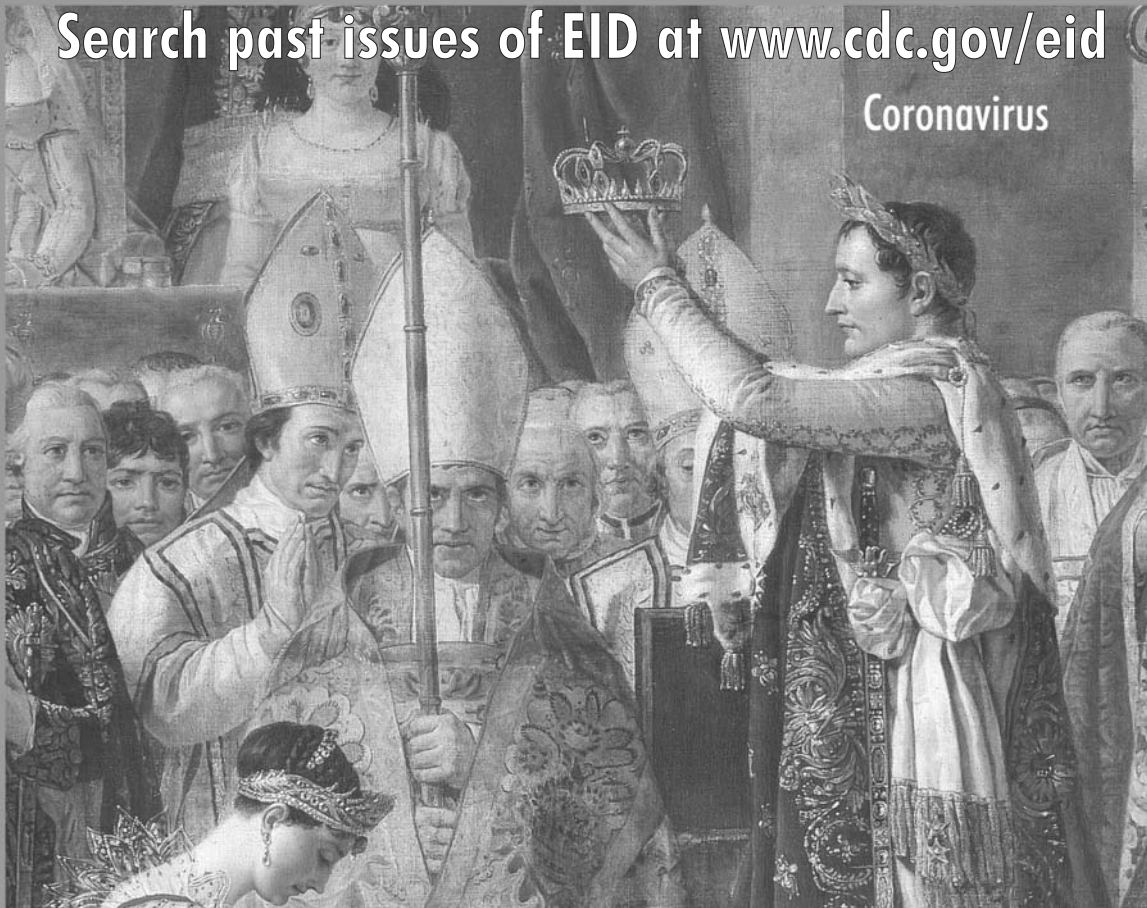
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Coronavirus



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# Inhibition of SARS Coronavirus Infection In Vitro with Clinically Approved Antiviral Drugs

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Bing Lim,\* and Lawrence W. Stanton\*

Severe acute respiratory syndrome (SARS) is an infectious disease caused by a newly identified human coronavirus (SARS-CoV). Currently, no effective drug exists to treat SARS-CoV infection. In this study, we investigated whether a panel of commercially available antiviral drugs exhibit in vitro anti-SARS-CoV activity. A drug-screening assay that scores for virus-induced cytopathic effects on cultured cells was used. Tested were 19 clinically approved compounds from several major antiviral pharmacologic classes: nucleoside analogs, interferons, protease inhibitors, reverse transcriptase inhibitors, and neuraminidase inhibitors. Complete inhibition of cytopathic effects of SARS-CoV in culture was observed for interferon subtypes,  $\beta$ -1b,  $\alpha$ -n1,  $\alpha$ -n3, and human leukocyte interferon  $\alpha$ . These findings support clinical testing of approved interferons for the treatment of SARS.

Severe acute respiratory syndrome (SARS) (1,2) is an infectious disease caused by a newly identified human coronavirus (SARS-CoV) (3,4). The disease can produce severe pneumonia with a reported fatal outcome of 15% to 20%. Currently, no effective drug exists to treat SARS-CoV infection (5). The urgency of the outbreak has led to the empiric use of broad-spectrum antibiotics and antiviral agents in affected patients in several countries (6–12). Intensive efforts are under way to gain more insight into the mechanisms of viral replication, in order to develop targeted antiviral therapies and vaccines. Developing effective and safe vaccines and chemotherapeutic agents against SARS CoV, however, may take years.

The recent epidemic has shown that knowledge is lacking regarding the clinical management and treatment of infected patients. Ribavirin (6–12), oseltamivir (8–10), foscarnet (8), intravenous immunoglobulin (8), and other agents have been used to treat patients. Preliminary results

from in vitro testing indicate that ribavirin concentrations that inhibit other viruses sensitive to ribavirin do not inhibit replication or cell-to-cell spread of the SARS-CoV (5). However, the U.S. Centers for Disease Control and Prevention concluded that further in vitro testing of antiviral drugs on other coronavirus isolates and more information on the clinical outcome of patients treated with ribavirin or other antiviral drugs in controlled trials is needed (5).

The aim of this study was to investigate whether a panel of currently available antiviral agents exhibit in vitro anti-SARS-CoV activity. Three general antiviral strategies are generally found (13): 1) direct antiviral effects, 2) inhibition of viral entry and replication at the cellular level by targeting virus-related processes, and 3) enhancement of host immune response. A total of 19 drugs approved for clinical use in the treatment of viral infections were tested in this study. They are representative compounds from major antiviral pharmacologic classes that are currently commercially available: nucleoside analogs, interferons, protease inhibitors, reverse transcriptase inhibitors and neuraminidase inhibitors.

A cell-based assay utilizing cytopathic endpoints (CPE) was set up using Vero E6 cells to screen these antiviral compounds. SARS-CoV has been shown to infect Vero E6 cells, an African green monkey kidney cell line (3), and this remains the only in vitro model of SARS-CoV infection. The initial screen was followed by a plaque reduction assay to determine the 50% effective concentration ( $EC_{50}$ ) of compounds showing positive results. These experiments allow rapid screening of commercially available antiviral agents, enabling those with in vitro evidence of activity to move expeditiously into clinical studies, since safety and pharmacokinetic information in humans is already available for other disease indications.

Here we report that certain interferon subtypes exhibit in vitro inhibitory activity against SARS-CoV and are candidates for follow-up studies in animal models and patients to determine their efficacy in vivo.

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## Materials and Methods

### Selection and Preparation of Drugs

To rapidly identify a pharmacologic agent that could be used to treat SARS, a collection of antiviral drugs was tested against SARS-CoV, the etiologic agent of the atypical pneumonia. To investigate a wide spectrum of potential molecular targets, we decided to cover the entire pharmacologic range of commercially available antiviral agents, including agents not expected to be active against coronaviruses. Information on antiviral drugs provided here was obtained from prescribing information sheets or from communications with the manufacturer.

Nucleoside analogues are a diverse class of compounds; in general, they inhibit viral RNA or DNA polymerases or other enzymes, interfering with nucleic acid synthesis. In this study, the selected compounds that target DNA viruses such as herpes simplex virus (HSV) and varicella-zoster viruses (VZV) were acyclovir, ganciclovir, and foscarnet. Ribavirin has activity against a range of DNA and RNA viruses; in different cell lines, ED<sub>50</sub> ranges from 1 to 100 µg/mL. Antiretroviral (HIV) drugs include reverse transcriptase (RT) inhibitors and protease inhibitors. Selected HIV nucleoside RT inhibitors studied were zidovudine and lamivudine, while HIV protease inhibitors studied were indinavir, nelfinavir, and saquinavir. The third group of antivirals studied were the neuraminidase inhibitors, both commercially available preparations, zanamivir and oseltamivir were used in this study. Interferons were the next major class of antivirals studied. Various subtypes of interferon  $\alpha$  (2a, 2b, n1, and n3, human leukocyte) and  $\beta$  (1a and 1b) were used. Amantadine, an old antiviral compound, was also studied. Different terms have been used to express antiviral activity, namely, EC<sub>50</sub>, 95% effective concentration (EC<sub>95</sub>), and 50% inhibitory concentration (IC<sub>50</sub>); Table 1 illustrates the range of activity against selected viruses.

Tenfold dilutions of the drug were tested to cover a broad range of concentrations above and below inhibitory dosages as reported by the manufacturer for other viral-host combinations. Compounds already present in aqueous injections were made up to volume by using Hank's buffered saline solution. For tablet and capsule formulations with soluble active ingredients, the outer coat was removed wherever applicable, and the preparation was ground in a mortar and pestle. The contents were dissolved in water, vortexed, and centrifuged thereafter at 3,000 g. The required volume was pipetted from the supernatant and diluted accordingly. When the active ingredients were insoluble in water (nelfinavir and saquinavir), the contents were dissolved in dimethylsulphoxide (DMSO); care was taken to ensure that the final concentration of DMSO in the dilutions would not exceed 1%. For plaque assays, fivefold

Table 1. Examples of inhibitory concentrations of antiviral drugs against selected viruses<sup>a</sup>

Compound	IC <sub>50</sub>	Virus
Foscavir	50–800 µmol/L	Cytomegalovirus
	5–443 µmol/L	Herpes simplex mutants
Acyclovir	0.01–13.5 µg/mL	Herpes simplex virus and varicella-zoster virus
Cymevene	0.02–3.48 µg/mL	Laboratory strains or clinical isolates of cytomegalovirus
Ribavirin	1–25 µg/mL	Influenza
	25–100 µg/mL	HIV and other retroviruses
	3.2–50 µg/mL (MIC)	Herpes and poxviruses suppression
Lamivudine	0.0006–0.034 µg/mL	HIV
Zidovudine	0.003–0.013 µg/mL	HIV
Fortovase	1–30 nmol/L	HIV
Viracept	7–196 nM (EC <sub>95</sub> )	HIV
Crixivan	25–100 nmol/L	HIV
Relenza	0.005–16 µmol	Influenza virus
Tamiflu	0.0008 µM–>35 µmol	Influenza virus
Amantadine	0.1–25 (ED <sub>50</sub> )	Influenza virus

<sup>a</sup>IC<sub>50</sub>, 50% inhibitory concentration; EC<sub>95</sub>, 95% effective concentration; ED<sub>50</sub>, 50% effective dose.

drug dilutions were prepared by using growth media as specified below.

### SARS-CoV Production and Infection

Vero E6 cells (American Type Culture Collection, Manassas, VA) were propagated in 75 cm<sup>2</sup> cell culture flasks in growth medium consisting of medium 199 (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel). SARS-CoV 2003VA2774 (an isolate from a SARS patient in Singapore), which has been previously sequenced (14), was propagated in Vero E6 cells. Briefly, 2 mL of stock virus was added to a confluent monolayer of Vero E6 cells and incubated at 37°C in 5% CO<sub>2</sub> for 1 h; 13 mL of medium 199 supplemented with 5% FCS was then added. The cultures were incubated at 37°C in 5% CO<sub>2</sub>, and the supernatant was harvested after 48 h; in  $\geq 75\%$  of cultures, inhibition of CPE (3+) in each well was observed with an inverted microscope. The supernatant was clarified at 2,500 rpm and then divided into aliquots, placed in cryovials, and stored at –80°C until use.

### Virus Handling and Titration

All virus culture and assays were carried out in the biosafety level-3 laboratory at the Environmental Health Institute, according to the conditions set out in Biosafety in Microbiological and Biomedical Laboratories (15). Virus titer in the frozen culture supernatant was determined by using a plaque assay. Briefly, 100 µL of virus in 10-fold serial dilution was added, in duplicates, to a monolayer of Vero E6 cells in a 24-well plate. After 1 h of incubation at



37°C in 5% CO<sub>2</sub>, the viral inoculum was aspirated, and 1 mL of carboxymethylcellulose overlay with medium 199, supplemented with 5% FCS, was added to each well. After 4 days of incubation, the cells were fixed with 10% formalin and stained with 2% crystal violet. The plaques were counted visually, and the virus titer in plaque-forming units per mL (PFU/mL) was calculated.

### Cytopathic Endpoint Assay

The protocol used was adapted from Al-Jabri et al. (16), and all drugs were tested in quadruplicate. Briefly, 100 µL of serial 10-fold dilutions of the drugs were incubated with 100 µL of Vero E6 cells, giving a final cell count of 20,000 cells per well in a 96-well plate. The incubation period was 1 h at 37°C in 5% CO<sub>2</sub>, except for the interferons, which were incubated overnight with the cells. Ten microliters of virus at a concentration of 10,000 PFU/well was then added to each of the test wells. The plates were incubated at 37°C in 5% CO<sub>2</sub> for 3 days and observed daily for CPE. The end point was the drug dilution that inhibited 100% of the CPE (CIA<sub>100</sub>) in quadruplicate wells. To determine cytotoxicity, 100 µL of serial 10-fold dilutions of the drugs was incubated with 100 µL of Vero E6 cells, giving a final cell count of 20,000 cells per well in a 96-well plate, without viral challenge. The plates were then incubated at 37°C in 5% CO<sub>2</sub> for 3 days and examined for toxicity effects by using an inverted microscope.

### Plaque Reduction Assay

Trypsinized Vero E6 cells were resuspended in growth medium and preincubated with interferons (serial fivefold dilution) in quadruplicate wells in 24-well plates. The next day, the medium was aspirated, and 100 µL of virus was added to each well at a titer of 100 PFU/well. After incubation for 1 h, the virus inoculum was aspirated, and a carboxymethylcellulose overlay containing maintenance medium and the appropriate interferon concentration was added. After 4 days' incubation, the plates were fixed and stained as described previously. The number of plaques was then counted visually, and the concentration of drug that inhibits 50% of plaques in each well (IC<sub>50</sub>) was deter-

mined. Results were plotted in Microsoft Excel, and a polynomial of order three was used to approximate the data and extrapolate IC<sub>50</sub> and IC<sub>95</sub> values.

## Results

### Cell-based Assay of SARS-CoV Infection

High titers of infectious SARS-CoV, originally derived from a respiratory sample of a SARS patient, were propagated on Vero E6 cells. The CPE of SARS-CoV on Vero E6 was evident within 24 hours after infection (Figure 1). SARS-CoV-infected cells display a CPE characterized by the appearance of rounded cells and the destruction of the monolayer.

### Antiviral Drug Activity

A collection of 19 antiviral drugs was tested in the SARS-CoV CPE inhibition assay (Table 2). The set of drugs tested included seven interferons, five nucleoside analogs, three protease inhibitors, two RT inhibitors, and two neuraminidase inhibitors. Complete inhibition of the CPE was observed for four of the seven interferons in the initial screen when very high viral challenge of 10<sup>4</sup> PFU/well and a high multiplicity of infection (MOI = 0.5) rate were used. Complete inhibition, expressed as CIA<sub>100</sub>, was observed for interferon β-1b (Betaferon) at 5,000 IU/mL, interferon α-n3 (Alferon) at 5,000 IU/mL, interferon α-n1 (Wellferon) at 250,000 IU/mL, and human leukocyte interferon α (Multiferon) at 500,000 IU/mL. Ribavirin also completely inhibited the CPE at 5,000 µg/mL (Table 3). None of the other drugs showed complete inhibition of CPE, even at the highest concentration of drug tested (Table 2).

Rebif (IFN-β-1a) showed slight inhibition of CPE at 250,000 IU/mL, but the inhibition was not complete at the screening virus load of 10,000 PFU/well. Likewise, Roferon (IFN-α-2a) showed slight, incomplete inhibition at 50,000 IU/mL. Because the criteria for ascertaining anti-SARS-CoV activity in this screen were set at 100% inhibition of CPE, and as high doses of interferons may result in severe clinical side effects, we chose to conduct further

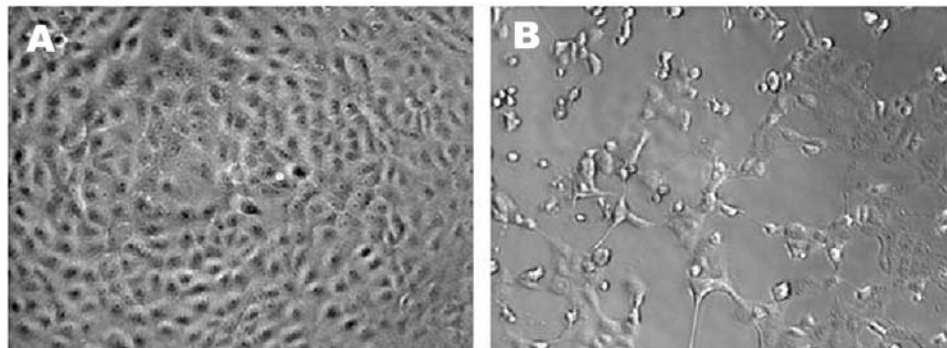


Figure 1. Microscopic appearance of control (A) and infected (B) Vero E6 cells, demonstrating cytopathic effects.

Table 2. Commercially available antiviral agents tested, source and starting concentration

Antiviral agent	Source	Highest concentration tested	Inhibition of cytopathic effect (CIA <sub>100</sub> )
<b>Interferons</b>			
Interferon $\alpha$ -2a (Roferon)	Roche	100,000 IU/mL	No
Interferon $\alpha$ -2b (Intron A)	Schering-Plough	500,000 IU/mL	No
Interferon $\alpha$ -n1 (Wellferon)	GlaxoSmithKline	500,000 IU/mL	Yes
Interferon $\alpha$ -n3 (Alferon)	Hemispheryx	10,000 IU/mL	Yes
Interferon $\beta$ -1a (Rebif)	Serono	500,000 IU/mL	No
Interferon $\beta$ -1b (Betaferon)	Schering AG	100,000 IU/mL	Yes
<b>Nucleoside analogs</b>			
Acyclovir	Faulding	1,000 $\mu$ g/mL	No
Ganciclovir (Cymevene)	Roche	50,000 $\mu$ g/mL	No
Ribavirin	ICN Pharma	10,000 $\mu$ g/mL	Yes
<b>Protease inhibitors</b>			
Indinavir (Crixivan)	Merck	100 $\mu$ mol/L	No
Nelfinavir (Viracept)	Roche	10,000 nmol/L	No
Saquinavir (Fortovase)	Roche	10,000 nmol/L	No
<b>Reverse transcriptase inhibitors</b>			
Lamivudine (Epivir)	GlaxoSmithKline	1,000 $\mu$ mol/L	No
Zidovudine (Retrovir)	GlaxoSmithKline	1,000 $\mu$ g/mL	No
<b>Neuraminidase inhibitors</b>			
Oseltamivir (Tamiflu)	Roche	10,000 $\mu$ mol/L	No
Zanamivir (Relenza)	GlaxoSmithKline	1,000 $\mu$ mol/L	No
<b>Other</b>			
Amantadine (Symmetrel)	Novartis	1,000 $\mu$ g/mL	No
Foscarnet (Foscavir)	AstraZeneca	8,000 $\mu$ mol/L	No

evaluations only in the interferons that showed complete inhibition from initial screen, namely, Wellferon, Multiferon, Betaferon, and Alferon.

Based upon results of the primary screen, the four active interferons and ribavirin were retested at two lower viral challenges,  $10^3$  and  $10^2$  PFU/ well. All four drugs again showed inhibitory effect, although the CIA<sub>100</sub> were dependent on viral loads (Table 3). At the lowest viral load the CIA<sub>100</sub> were 5 IU/mL for both interferon  $\beta$ -1b (Betaferon) and human leukocyte interferon  $\alpha$  (Multiferon); and 50 and 250 IU/mL for interferon  $\alpha$ -n3 (Alferon) and interferon  $\alpha$ -n1 (Wellferon), respectively. No cytotoxicity of the interferons was observed at or near inhibitory concentrations. Ribavirin showed inhibitory activity at all three viral loads, but only at high concentrations of the drug, 0.5–5 mg/mL. At high concentrations of ribavirin (0.2–1 mg/mL) cytotoxic effects were observed on VeroE6 cells, as has been reported for other cell types (17,18). As such, we consider ribavirin to be inactive against SARS-CoV.

A plaque reduction assay format with 100 PFU of SARS-CoV (MOI = 0.0005) was conducted to determine the IC<sub>50</sub> for Betaferon, Alferon, and Multiferon, the three compounds that showed greatest potency for inhibition of CPE. Additional supply was not available for testing interferon  $\alpha$ -n1 (Wellferon), as production of this drug has been discontinued. Cells were preincubated for 15 h with fivefold dilutions of drug. Viral-induced plaques, which

developed in 3 days, were counted to determine the inhibitory effect of the drugs at various concentrations. All three interferon preparations displayed a dose-dependent inhibition of SARS-CoV plaque formation in this assay (Figure 2). The IC<sub>50</sub> and IC<sub>95</sub> were determined to be 0.2 and 8 IU/mL for Betaferon, 0.8 and 200 IU/mL for Alferon, and 2 and 44 IU/mL for Multiferon.

## Discussion

Betaferon, Alferon, Multiferon, Wellferon, and ribavirin inhibited CPE in SARS-CoV-infected Vero E6 cells, in decreasing order of potency. Ribavirin, a drug widely used in initial efforts to manage SARS infections, inhibited CPE completely at 500–5,000  $\mu$ g/mL at virus loads of 100–10,000 PFU per well. The concentration range observed is much higher than concentrations that inhibit other viruses (respiratory syncytial virus, ED<sub>50</sub> 2–8  $\mu$ g/mL, HIV or resistant strains of rhinovirus, 50–100  $\mu$ g/mL), including viruses that were tested on Vero cells (West Nile virus, New York isolate 178  $\mu$ g/mL, and Uganda isolate 41  $\mu$ g/mL) (19). In addition, the CPE

Table 3. Complete inhibition of cytopathic effect (CIA<sub>100</sub>) at different virus titers

Virus load (PFU/well)	Ribavirin ( $\mu$ g/mL)	Wellferon (IU/mL)	Betaferon (IU/mL)	Alferon (IU/mL)
10,000	10,000	500,000	10,000	10,000
1,000	10,000	5,000	1,000	1,000
100	1,000	500	10	100

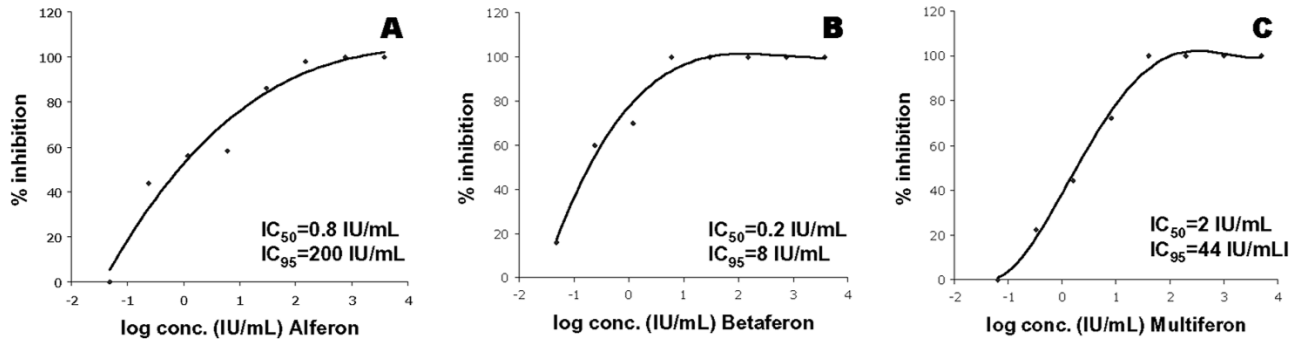


Figure 2. Dose-response curves for Alferon (A), Betaferon (B), and Multiferon (C) as determined by plaque reduction assays.  $IC_{50}$  (50% inhibitory concentration) and  $IC_{95}$  (95% inhibitory concentration) values were calculated by using the fitted functions describing the curves.

inhibitory concentrations obtained in this study were above the cytotoxic concentration range against Vero cells. The 50% cytotoxic dose ( $CD_{50}$ ) on various cell lines has been reported to be approximately 200–1000  $\mu\text{g}/\text{mL}$  of ribavirin (17,18). We observed slight cytotoxicity by microscopic examination of the cells, making it difficult to accurately obtain in vitro efficacy data against SARS-CoV. It appears that due to the low activity of ribavirin in vitro, inhibitory doses may not be achievable clinically. It is possible that ribavirin would be more effective in combination with interferons. Combination therapy with ribavirin and interferon  $\alpha$  has now become standard treatment for chronic hepatitis C (20–22). Additionally, we have tested the effect of ribavirin and Betaferon in combination (range of concentration of ribavirin, 1–100  $\mu\text{g}/\text{mL}$ ; range of concentration of Betaferon, 0.1–10 IU/mL). At 1,000 PFU, this combination did not demonstrate observable synergistic inhibitory effect against SARS-CoV.

This study describes in vitro activity of four interferon subtypes against the SARS-CoV. Interferons have been used as anticancer and antiviral agents, in particular, for treating hepatitis B and C infections. Various groups have reported the clinical benefit of intranasally administered interferon  $\alpha$  in human volunteers before and after inoculation with non-SARS coronaviruses (23–25). The antiviral activity of interferons is mediated by direct effects on infected cells or by modulating an immune response (26). Interferons interact with specific surface cell receptors, leading to production of interferon-stimulated gene products such as 2'5'-oligoadenylate synthase and protein kinase PKR (27).

In SARS-CoV infection, a convenient starting point for the use of interferons against a SARS-CoV infection would be the usual clinical doses for the treatment of hepatitis B or C. Common clinical dosages for interferon  $\alpha$  range from 3 to 5 million IU three times a week to 5 million IU daily. For interferon  $\beta$ , data regarding efficacy in the treatment of hepatitis C are conflicting, and interferon

$\beta$  (at doses of 3 to 6 million IU three times weekly) is usually only used in the treatment of infections in patients whose condition no longer responds to other therapies. Plasma levels of interferons administered through the subcutaneous route are usually low with correspondingly short half-lives. In view of their mechanism of action, absolute serum levels may not be meaningful as a measure of the biologic activity of interferons, compared to the induction of cellular products such as 2'5' oligoadenylate synthase.

Interferon activity varies among different cell types (28,29), however. Specific interferon subtypes which inhibit SARS-CoV in Vero cells may not necessarily have the same effect in other cells; the converse may also be true—that those drugs that are negative in Vero cells may be effective in other cell types. We are currently identifying other in vitro models of SARS-CoV infection that will enable us to address cell-type specific drug effects. Also, interferon subtypes exhibited different activity against SARS-CoV in this study. The mechanism for the difference in activity is unknown. Among the products tested, the source of interferon and amount of glycosylation differ. Some preparations were derived from human lymphoblastoid or leukocyte cells, while others were recombinantly produced in *Escherichia coli* or mammalian cell culture. We do not know the importance of this observation with respect to possible antiviral mechanisms of the interferons against SARS-CoV or potential clinical implications of these differences.

This study describes rapid screening of commercially available compounds for extension into in vivo research. Evidence of activity and data from in vitro studies, however, cannot be easily correlated with clinical performance but rather present promising candidates for follow-up studies. Definite recommendations on anti-SARS-CoV activity of compounds in humans can only be made in the in vivo setting. In conclusion, interferon  $\beta$ -1b,  $\alpha$ -n1,  $\alpha$ -n3, and human leukocyte interferon  $\alpha$  exhibit antiviral activity in an

vitro model and are potential drugs for in vivo research and clinical management of SARS-CoV infection.

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# SARS Transmission, Risk Factors, and Prevention in Hong Kong

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We analyzed information obtained from 1,192 patients with probable severe acute respiratory syndrome (SARS) reported in Hong Kong. Among them, 26.6% were hospital workers, 16.1% were members of the same household as SARS patients and had probable secondary infections, 14.3% were Amoy Gardens residents, 4.9% were inpatients, and 9.9% were contacts of SARS patients who were not family members. The remaining 347 case-patients (29.1%) had undefined sources of infection. Excluding those  $\leq 16$  years of age, 330 patients with cases from "undefined" sources were used in a 1:2 matched case-control study. Multivariate analysis of this case-control study showed that having visited mainland China, hospitals, or the Amoy Gardens were risk factors (odds ratio [OR] 1.95 to 7.63). In addition, frequent mask use in public venues, frequent hand washing, and disinfecting the living quarters were significant protective factors (OR 0.36 to 0.58). In Hong Kong, therefore, community-acquired infection did not make up most transmissions, and public health measures have contributed substantially to the control of the SARS epidemic.

As of June 11, 2003, a total of 1,755 probable SARS cases were reported in Hong Kong (1). Some of the sources of SARS transmission are unknown. For instance, the first major SARS outbreak occurred in the Prince of Wales Hospital in March 2003, and 138 probable cases were reported from March 11 to March 25, 2003 (2). Another major outbreak occurred in the Amoy Gardens housing estate on approximately March 26, 2003, and a total of 321 residents were affected (3). A total of 381 hospital workers were affected as of May 29, 2003 (4). Other sources of infection are possible. Some inpatients were cross-infected by SARS case-patients, who were hospitalized for reasons other than SARS; others may have contracted the disease through known contacts with other SARS patients. The rest of the community-acquired case-patients contracted the diseases through less defined sources. The distributions of the "known" and "undefined"

sources of infection have not been reported. Such an initiative would help assess the infectivity and modes of transmission of the virus in the community setting.

Also, reports that public health measures, such as wearing masks, frequent hand washing, avoidance of crowded places, disinfection of the living quarters had been practiced by most of the Hong Kong population during the SARS outbreak ( $>90\%$ ) (5). The efficacy of widespread use of masks was controversial (6), and evaluating the efficacy of such measures in controlling the epidemic is important.

Our study had two objectives. First, we sought to delineate the distribution of different sources of transmission of the SARS cases in Hong Kong. The number of cases with known and undefined sources was determined. Patients with known sources included those who were hospital workers, those who lived in the Amoy Gardens Estate, those who were probable secondary cases within a household (i.e., those with another household member who had SARS with an earlier date of onset), those who were inpatients and were cross-infected by other inpatients, and those persons who had contact with another SARS patient (who was not one of their household members) before the onset of fever. For the remaining cases, the virus was contracted through undefined sources.

The second objective was to identify the undefined source group. A number of hypotheses were tested to identify relevant risk and protective factors associated with contracting the disease. Risk factors were related to visiting places of potentially high risk and meeting at-risk people. Preventive factors were related to public health measures for prevention.

## Methods

The study population comprised all probable SARS patients whose cases were reported to the Department of Health on or before May 16, 2003 ( $n = 1,690$ ). The SARS case definition criteria, used by Hong Kong Hospital Authority, is as follows: radiographic evidence of infiltrates consistent with pneumonia and current temperature  $>38^{\circ}\text{C}$  or a history of such at any time in the preceding 2 days, and

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at least two of the following: history of chills in the past 2 days, new or increased cough, breathing difficulty, general malaise or myalgia, typical signs of consolidation, and known exposure. These criteria for cases are equivalent to those in the World Health Organization's case definition for probable SARS cases (7).

### Data Collection

Telephone numbers, as well as some demographic and clinical background information, for all probable SARS case-patients in Hong Kong (identified on or before May 16, 2003 [n = 1,690]) were obtained from the Department of Health. A team of trained interviewers called all these numbers, briefed the person answering the phone about the nature of the study, and invited their household to join the study. Informed consent was obtained directly from the respondents. The number of SARS patients in the household was ascertained, and the interviewer identified the index patient, the person who had the earliest date of onset of fever if the household had more than one SARS patient. The rest of the SARS patients, those with later onset of illness, were considered as having probable secondary or tertiary cases. When a household had had two or more SARS patients with the same fever onset date (11 households), both were treated as index patients rather than as having probable secondary cases. The information obtained was cross-checked with that obtained from the SARS registry. Ethics approval was obtained from the Ethics Committee of the Chinese University of Hong Kong.

The study was conducted from April 4, 2003, through June 10, 2003. Of the 1,690 probable SARS case-patients reported in Hong Kong as of May 16, a total of 1,214 (72%) SARS case-patients from 996 households were covered by our study. Of the remaining 476 case-patients not covered by this study, 140 case-patients (8.2%) did not have a correct telephone number, 163 (9.6%) could not be contacted after at least five attempts, 163 (9.6%) declined to participate, and 10 (0.6%) were either not in Hong Kong or could not communicate in Chinese or English.

### Study Design

The study is part of a project that also includes an investigation of the secondary attack rate of household members. For the first part of this study, the index case-patients were asked whether they were hospital workers, inpatients before contracting SARS, or residents of the Amoy Gardens. The other respondents were asked whether onset of fever occurred within 10 days of contact with a SARS patient. These four types of SARS cases were classified into the known sources group. The rest of the index case-patients were classified into the undefined source group. In the second part of the study, a 1:2 matched case-control study was conducted for the undefined source

group to identify risk and preventive factors associated with SARS transmission in the community setting.

Adults  $\geq 16$  years of age were included in the case-control study (17 case-patients were removed from the analysis). Potential geographically related risk factors studied included whether the case-patient had visited (but not lived in) Amoy Gardens, Prince of Wales Hospital, other hospitals or clinics, or crowded places within 10 days before onset of fever. Other risk factors were related to contact with other groups of people during the same reference period, including medical personnel, hospital visitors, and persons with influenzalike symptoms (who were not SARS case-patients). A number of protective factors were related to relevant public health measures, including the frequency of using a face mask in public venues, the frequency of washing hands each day, and disinfection of living quarters thoroughly during the same period. The same questions were asked to the control group, which was recruited by a random telephone survey. Members of the control group were matched for age and sex with the case-patient.

The reference period was the same as that of the matched case-patient. Random telephone numbers were selected from up-to-date local telephone directories. Interviews were conducted in the evening to avoid over-representing those who were not working during the daytime. At least three calls were made before an unanswered call was considered as a noncontact. Informed consent was obtained before the interviews were conducted. Almost all case-patients were interviewed within 14 to 28 days after their onset of fever, and the control group was interviewed accordingly. When a participant was unable to answer the questionnaire, a proxy, who was most familiar with the family situation, was interviewed.

### Data Analyses

For the case-control study, odds ratios (OR) were first examined by using univariate logistic regression models. The significant univariate variables were then entered as input for the multivariate forward conditional logistic regression analysis; p values  $< 0.05$  were statistically significant. SPSS for Windows Release 11.0.1 (SPSS Inc., Chicago, IL) was used to analyze the data.

## Results

### Cases with Known Sources of Transmission

Of the 1,214 probable SARS cases covered by this study, 22 questionnaires (1.8%) were incomplete and did not allow us to classify the respondents into groups according to source of transmission. The rest (n = 1,192) were analyzed. A total of 192 (16.1%) had probable cases of secondary or tertiary household transmission (Table 1) (i.e.,

Table 1. Distribution of 1,214 severe acute respiratory syndrome cases covered by the study<sup>a</sup>

	n	% <sup>b</sup>
Known sources	727	61.0
Probable secondary/tertiary household infection	192 <sup>d</sup>	16.1
Hospital care workers	317 <sup>d</sup>	26.6
Amoy Gardens residents	170 <sup>d</sup>	14.3
Inpatients	58 <sup>d</sup>	4.9
Unknown sources		
Contacted SARS patient(s) within 14 days before onset of fever <sup>c</sup>	118	9.9
Undefined sources	347	29.1
Visited Amoy Gardens	54 <sup>d</sup>	4.5
Visited PWH <sup>e</sup>	12 <sup>d</sup>	1.0
Visited other hospitals or clinics	142 <sup>d</sup>	11.9
Visited an affected country	45 <sup>d</sup>	3.8
None of the above	124	10.4

<sup>a</sup>Incomplete information for 22 cases; complete information for 1,192 cases.  
<sup>b</sup>Calculated based on complete data.  
<sup>c</sup>These patients did not belong to the categories listed under known sources.  
<sup>d</sup>These categories are not mutually excluded, i.e., a respondent may be exposed to more than one category.  
<sup>e</sup>PWH, Prince of Wales Hospital.

another household member had SARS but fever onset occurred earlier). All the names were verified as being reported to the SARS registry. Another 317 of 1,192 (26.6%) cases were hospital workers; 170 (14.3%) lived in the Amoy Gardens; 58 (4.9%) were inpatients who had been hospitalized for diseases other than SARS and kept on wards with SARS patients. Most infected inpatients were long-term chronic patients and had been hospitalized for >2 weeks before having SARS symptoms. These patients were likely to have been cross-infected. A total of 727 case-patients belonged in one of the four categories (61% of 1,192 cases). Another 118 (9.9%) had come into contact with a SARS patient within a 10-day period before onset of fever. For 347 (29.1%) participants, the source was undefined; these participants were included in the case-control analysis. After excluding 17 case-patients ≤16 years of age, 330 participants were included in the case-control study.

### Univariate Case-Control Analysis

Of the 330 patients with an undefined source of infection, 48% were men and 52% were women. The mean age of the patient group was 47.1 years for both the male and female case-patients (standard deviation [SD] 18.8 and 19.9, respectively,  $p > 0.05$ ,  $t$  test). The percentage of participants in the undefined source group in the three periods of the epidemic (before March 25, 2003, from March 26 through April 10, and after April 10) were 24.2%, 36.1%, and 43.5%, respectively.

Members of the patient group were more likely than the control group to have visited mainland China (12.7% vs. 6.5%,  $p < 0.005$ ). One patient had visited Taiwan,

another patient had visited Singapore, two controls had visited Taiwan, and none of the controls had visited Singapore (Singapore and Taiwan were listed as affected areas during the study period). Similarly, patients were also more likely than controls to have visited the Amoy Gardens (15% vs. 2%, OR = 9.10,  $p < 0.005$ ) (keeping in mind that those who lived in the Amoy Gardens had already been removed from the analysis); patients were more likely than the controls to have visited the Prince of Wales Hospital (3.6% vs. 0.5%, OR = 8.27,  $p < 0.005$ ) or other hospitals or clinics (40.7% vs. 17.0%, OR = 3.36,  $p < 0.005$ ) (Table 2). A total of 212 cases of the undefined source group had visited at least one of the above-mentioned categories of places. Frequency of visiting crowded places was, however, not significant in the univariate analysis (21.91% vs. 20.8%, OR = 1.07,  $p > 0.05$ ).

Members of the case-patient and control groups were not statistically different in the percentage of having come into contact with someone with influenzalike symptoms (those having made contacts with SARS patients were already removed, 9.0% vs. 6.4%, OR = 1.42,  $p > 0.05$ ). The two groups were also not different in the likelihood of having social contact with someone who had visited a hospital (8.2% vs. 5.2%, OR = 1.66,  $p > 0.05$ ) or having social contact with medical personnel (7.6% vs. 8.6%, OR = 0.87,  $p > 0.05$ ). Also patients were not more likely to have a known SARS patient living in the same housing estate, after Amoy Gardens patients had already been removed from the analysis (such data were made available to the public by the government after April 12, 2003) (8).

Furthermore, matching for the reference period, members of the case group were less likely than members of the control group to have frequently worn a face mask in public venues (27.9% vs. 58.7%, OR = 0.36,  $p < 0.005$ ), to have been washed their hands >10 times a day (18.4% vs. 33.7% OR = 0.44,  $p < 0.005$ ), and to have disinfected their living quarters thoroughly (46.6% vs. 74.5%, OR = 0.30,  $p < 0.005$ ).

### Multivariate Analysis

When all the variables that were significant in the univariate analysis were used as input for the multivariate stepwise conditional logistic regression analysis, the results show that among the 330 patients with undefined sources, the following were significant risk factors: having visited mainland China (OR = 1.95,  $p = 0.020$ , Table 2), having visited the Amoy Gardens (OR = 7.63,  $p < 0.001$ ), having visited the Prince of Wales Hospital (OR = 7.07,  $p = 0.009$ ), and having visited other hospitals or clinics (OR = 3.70,  $p < 0.001$ ) during the reference period. On the other hand, using a mask frequently in public places (OR = 0.27,  $p < 0.001$ ), washing one's hands >10 times a day (OR = 0.58,  $p = 0.008$ ), and disinfecting the living quarters thor-

Table 2. Preventive measures and risk factors reported by cases and controls

Factors	Case <sup>b</sup>	Control <sup>c</sup>	Matched univariate OR (95% CI)	Matched multivariate OR (95% CI)	p value <sup>d</sup>
% visited mainland China (reference=no)	12.7	6.5	2.09 (1.33 to 3.27) <sup>e</sup>	1.95 (1.11 to 3.42)	0.020
% visited PWH (reference=no)	3.6	0.5	8.27 (2.32 to 29.49) <sup>e</sup>	7.07 (1.62 to 30.75)	0.009
% visited other hospitals/clinics (reference=no)	40.7	17.0	3.36 (2.49 to 4.54)	3.70 (2.54 to 5.39)	<0.001
% visited Amoy Gardens (reference=no)	15.5	2.0	9.10 (4.87 to 17.00) <sup>e</sup>	7.63 (3.77 to 15.43)	<0.001
% visited crowded places frequently (reference=occasionally/seldom/no)	21.9	20.8	1.07 (0.76 to 1.50) NS	-	-
% contacted someone with fever or influenza (reference=no)	9.0	6.4	1.42 (0.87 to 2.32) NS	-	-
% social contact with someone who visited a patient in a hospital (reference=no)	8.2	5.2	1.66 (0.96 to 2.85) NS	-	-
% social contact with medical personnel (reference=no)	7.6	8.6	0.87 (0.52 to 1.44) NS	-	-
% had a SARS case in the housing estate (reference=no)	6.6	8.5	0.76 (0.44 to 1.31) NS	-	-
% disinfected the living quarters thoroughly (reference=no)	46.6	74.5	0.30 (0.23 to 0.39) <sup>e</sup>	0.41 (0.29 to 0.58)	<0.001
Wore a mask in public places frequently (reference=occasionally /seldom/no)	27.9	58.7	0.27 (0.20 to 0.37) <sup>e</sup>	0.36 (0.25 to 0.52)	<0.001
Washed hands 11 or more times per day (reference=1–10 times/day)	18.4	33.7	0.44 (0.31 to 0.63) <sup>e</sup>	0.58 (0.38 to 0.87)	0.008

<sup>a</sup>N.S., not significant; OR, odds ratio; CI, confidence interval; PWH, Prince of Wales Hospital; -, not used by the multivariate analyses. The reference time period was the 10 days before the date of the patient's onset of fever.

<sup>b</sup>n = 330.

<sup>c</sup>n = 660.

<sup>d</sup>p values for multivariate OR.

<sup>e</sup>p < 0.005.

oughly (OR = 0.41,  $p < 0.001$ ) during the reference period were significant protective factors (Table 2).

### Undefined Cases

After removing those case-patients who may have contracted SARS after visiting the Amoy Gardens, the Prince of Wales Hospital, other hospitals, or an affected country, including mainland China, Singapore, and Taiwan (212 cases of the 330 cases), 118 cases remained undefined. They were likely to be community-acquired cases of unknown sources of transmission. When univariate and multivariate conditional logistic regression analyses were repeated for the 118 cases with undefined sources (after 212 patients who had visited some particular places that were associated with risk for transmission were removed from the analysis) and their controls ( $n = 236$ ), similar results were obtained. The three public health variables—frequently wearing a mask in public places (adjusted OR = 0.36,  $p < 0.001$ ), washing hands >10 times a day (adjusted OR = 0.44,  $p = 0.008$ ), and disinfecting the living quarters thoroughly (adjusted OR = 0.36,  $p < 0.001$ )—remained significant protective factors. Again, similar to the results of the previous analysis applied to the 330 cases, the other five variables (visiting crowded places, having contact with someone with influenzalike symptoms, having social contact with hospital visitors, having social contact with medical workers, and living with in the same housing estate as other SARS case-patients) were not significant risk factors.

### Discussion

Of the 1,192 participants in this study, approximately 16.1% had probable secondary or tertiary transmission occurring within the household, 26.6% were hospital workers with nosocomial infections, 14.3% were Amoy Gardens patients, and 4.9% were cross-infected inpatients. In 9.9%, SARS might have been contracted when the participant came in contact with a SARS patient who was a nonhousehold member, which may have occurred in a hospital or community setting. SARS may have developed in 18.7% after they visited Amoy Gardens, hospitals or clinics, or affected countries. This computation leaves 9.9% as community-acquired cases of an unknown source.

The percentage of patients related to Amoy Gardens (someone who lived there or visited there) is 18.8% (224/1,192). The percentage of patients with a hospital connection (hospital workers, inpatients, and visitors) is 44% (525/1,192). The proportion of unknown community-acquired SARS infection among all SARS cases in this study was considerably lower than the proportion of nosocomial infection, which suggests that preventing hospital outbreaks is essential.

Of the 330 undefined transmissions, 44.2% of the transmissions occurred through hospital visitors. Another study on household transmission also indicated that hospital visits were a significant risk factor for predicting household secondary infection (9). Therefore, the severity of future outbreaks, if any, would depend on the ability of the hos-



pital system to control hospital cross-infection and infection of visitors.

Visits to mainland China were associated with SARS transmission, even after adjusting for other variables. Cross-border transmission played a role in the epidemic; although the absolute percentage is not high among the 1,192 case-patients (3.6% or 43/1,192), it is substantially larger among the undefined source group (12.4%). With a case-control design, we could not establish whether this 14.4% was associated with an inflated risk. Cross-border communication and prevention, such as those set in place (temperature screening and health declaration), need to be enforced strictly and consistently. Almost 70% of the 43 participants who visited mainland China had fever onset on or before April 1 (i.e., the early phase of the epidemic) (5). None of them had onset after May 3, which is understandable as visiting mainland China was perceived as a high risk by the general public in the late phase of the epidemic (5).

The variables related to social contacts (with medical personnel or hospital visitors, with persons with influenza-like symptoms, and with persons living in a housing estate with a reported SARS patient) were not significant. These findings should be interpreted with caution. On one hand, these case-patients should not be stigmatized. On the other, the results may have been confounded because all SARS cases contracted this way were excluded from the analysis. However, confirming that these variables could not account for transmission of the undefined source cases can be useful.

Evidence does not indicate that frequent visits to crowded places were associated with a higher likelihood of community-acquired infection. This finding may remove panic that arose during the epidemic, and daily life need not change as much as it had. Hong Kong is a densely populated city, and it had a large number of SARS cases. The number of community-acquired cases in less populated cities should be much lower than that of Hong Kong. This finding should be interpreted with care as >90% of the general public wore face masks in public places, and >85% avoided visits to public places during the epidemic in Hong Kong (5). Although visiting the Amoy Gardens was a risk factor, Amoy Gardens might be the only place where such a large-scale SARS outbreak was attributable to contamination of the environment.

We now have some empirical evidence to suggest that wearing a face-mask frequently in public places, frequent handwashing, and disinfecting one's living quarter were effective public health measures to reduce the risk for transmission (adjusted OR 0.58 to 0.36). The effectiveness of mask use was controversial (6). In another study, the prevalence of these three public health preventive public health measures increased significantly from March 21,

2003, to April 1, 2003, (i.e., wearing masks 11.5%–84.3%; frequent hand washing 61.5%–95.1%; home disinfection 36.4%–80%) (5). These practices played an essential role in limiting the spread of the virus in the community in Hong Kong.

That disinfecting the living quarter is a strong protective factor has a particular relevance. The reason behind the significance is not completely clear. During the epidemic, the Hong Kong government released frequent announcements of public interest to promote home disinfection using 1:99 bleach water solutions. Most respondents who disinfected their living quarters were probably following the government's suggestion. Keeping in mind that probable secondary cases had already been removed from the analysis, such protective effect is not referring to the effects that disinfecting the quarter reduced the chance of secondary infection. Environmental contamination (suspected to be related to the sewage system) was reported in the Amoy Gardens, and similar environmental contamination probably did not occur in other places. Such contamination-related infections might be on a small scale and not been noticed. In such circumstances, home disinfection might reduce the risk for transmission. The finding suggests that, in addition to the droplet theory, the fomites theory could not be dismissed.

Our study has a few limitations as well as strengths. First, approximately 72% of all SARS case-patients were included in the study (excluding patients whose contact numbers were incorrect or not available; approximately 78% of those with a valid contact telephone number were included, and the refusal rate was about 10%). The sample size was reasonably large. Second, data were collected retrospectively. Most of the data were, however, collected from the participants within 1 month after onset of fever. Since contracting the disease is a major life event for the patient and family, they should be able to recall whether such factual and benchmark behaviors had been practiced.

The study also has strength of matching for age, sex, and reference time of the behaviors in question, so that both the case and control in a pair were referring to relevant behaviors that occurred within the same 10-day period before the date of onset of fever of the patient. Third, some questions, such as those about disinfection of households or visiting crowded places were nonspecific (the questions asked were "Whether your living quarter had been disinfected thoroughly" and "Whether you had visited crowded places"). Different participants might have defined the terms differently. Further, a number of patients were unable to answer the questions, and a household member who was "most familiar with the household situation" was invited to serve as a proxy. The responses obtained from these informants were compared to those obtained from the patients themselves, and no statistical

significance was obtained (p 0.199 to 0.854) to all variables, except for the variable about visiting the Amoy Gardens (p < 0.05).

One particular strength of the study in its evaluation of the three public health measures is that transmissions due to various known sources of infection had been removed as much as possible. In conclusion, the study shows that public health measures may have contributed substantially to the control of SARS epidemic in Hong Kong.

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# Myanmar Dengue Outbreak Associated with Displacement of Serotypes 2, 3, and 4 by Dengue 1

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In 2001, Myanmar (Burma) had its largest outbreak of dengue—15,361 reported cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), including 192 deaths. That year, 95% of dengue viruses isolated from patients were serotype 1 viruses belonging to two lineages that had diverged from an earlier, now extinct, lineage sometime before 1998. The ratio of DHF to DSS cases in 2001 was not significantly different from that in 2000, when 1,816 cases of DHF/DSS were reported and dengue 1 also was the most frequently isolated serotype. However, the 2001 ratio was significantly higher than that in 1998 (also an outbreak year) and in 1999, when all four serotypes were detected and serotypes 1, 2, and 3 were recovered in similar numbers. The large number of clinical cases in 2001 may have been due, in part, to a preponderance of infections with dengue 1 viruses.

Dengue is a disease caused by four serotypes of a flavivirus of the same name (1). Infection with these viruses may be inapparent, or it may result in disease varying in severity from a mild influenza-like illness to hemorrhagic fever and hypovolemic shock, which may be fatal if untreated (1). Dengue is an important source of illness and death in tropical nations, particularly in Southeast Asia and Central and South America (2). In 1998, a pandemic of dengue resulted in 1.2 million cases of dengue hemorrhagic fever (DHF) in 56 countries (3). In many countries in Asia where this disease is endemic, outbreaks occur in cycles of 3 to 5 years due, perhaps, to enhanced infection with one serotype caused by cross-reactive antibody produced in response to an earlier infection with a second serotype (4), rather than to climatic effects (5). Furthermore, the incidence of disease and its severity vary between primary and secondary infections and between

infections with different dengue virus serotypes (6–8). All four dengue virus serotypes circulate in countries in Southeast Asia from Myanmar to Indonesia (9–13), and no outbreaks caused by single virus serotypes, as have been seen in dengue non-endemic areas such as Cuba or Australia (14,15), have recently been reported. Phylogenetic studies have shown regular extinction of strains of dengue viruses in single locations and emergence of new strains (16,17), and it has been suggested that the appearance of more fit or more pathogenic viruses may occur as a result of immunologic selection during periods of intense transmission during outbreaks (18). While weak selective pressure on the envelope (E) protein gene of some dengue viruses is evident (19), the most extensive changes in virus genotypes appear to be due to recombination or to possible genetic bottlenecks (16,17,20). Given these observations, and the finite pool of hosts in most locations (humans and selected species of *Aedes* mosquitoes), perhaps it is surprising that greater competition between the four serologically related serotypes of dengue virus has not been observed, e.g., the complete exclusion of two or three serotypes from an ecological niche.

## Patients and Methods

### Serology

Acute- and, when possible, convalescent-phase serum samples were obtained from patients admitted to the Yangon Children's Hospital with a clinical diagnosis of DHF (1). A patient with a confirmed case of dengue fever was one who met any of the following criteria: 1) paired sera showed a fourfold or greater rise in hemagglutination inhibiting (HI) antibody titer against dengue virus (21); 2) a convalescent-phase serum sample produced an immunoglobulin (Ig) G reaction (titer equivalent to an anti-dengue virus HI titer of >2,560), an IgM reaction in a commercial "rapid" dengue test (22), or both; or 3) dengue

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virus was recovered by culturing 140  $\mu$ L of acute-phase serum on 25-cm<sup>2</sup> monolayers of C6–36 *A. albopictus* cells for 7 days. The serotype of the virus was determined by performing indirect immunofluorescence (23) on cells from the cultures of C6–36 cells used for virus isolation with flavivirus-, dengue-, or serotype-specific monoclonal antibodies (23,24).

### Phylogenetic Analyses

RNA was extracted from virus isolates with a commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, transcribed to cDNA, and amplified by polymerase chain reaction as described previously (24). The cDNA was purified and then sequenced on an automated sequencer (Applied Biosystems, Inc., Foster City, CA) (24). Nucleotide sequences were edited, compared, and analyzed with software (EclustalW, Ednapars, Ednadist, Ekitsch) from the Australian Genome Information Service (available from: <http://www.angis.org.au>). Additional nucleotide sequences used in the phylogenetic analyses are listed as country, year of isolation, dengue virus serotype, and GenBank accession number, i.e., (Sin[agapore]90D1, M87512; Abid[jan]99D1, AF298807; Mal[aysia]72D1-12, AF231721; Thai64D1, AF180817; Thai58D1, D10513; Phil[ippines]D1, D00503; Nauru74 D1, U88535; Mex[ico]83D1, D00504; Jam[aica]77D1, D00501; Japan43D1M, AB074760; Haw[aii]44D1, X76219; Lao96D1, AB003090; China 80D1, AF350498; Camb[odia]98D1, AF309641).

### Results

Dengue is endemic in Myanmar. Outbreaks have occurred in 3- to 5-year cycles of increasing magnitude since the first recorded outbreak in the country in 1970 (Table 1). The outbreak in 2001 (15,361 cases of DHF/dengue shock syndrome [DSS]) was the largest on record.

In 1998 and 1999, all four dengue virus serotypes (DENV-1–4) were recovered from patients in the Yangon Children's Hospital; DENV-1, -2, and -3 were recovered in approximately similar ratios each year (Table 2). In 2000 and 2001, no DENV-4 was recovered, and DENV-1 was recovered more frequently than any other serotype. In

2001, 95% of isolates were DENV-1. No significant difference ( $p \geq 0.05$ , chi-square test) was found in the rate of isolation of dengue viruses from seronegative serum samples for each of these years.

Accompanying the change in the relative proportions of dengue virus serotypes recovered from patients in the Yangon Children's Hospital was a significant change in the relative proportion of clinically diagnosed DHF and DSS cases (1998: 3,194 DHF, 1,402 DSS; 1999: 1,741 DHF, 601, DSS; 2000: 896 DHF, 224 DSS; 2001: 4,511 DHF, 1,105 DSS), i.e., DSS occurred in a smaller proportion of patients in 2000 and 2001 than in 1999 or 1998 ( $p \leq 0.01$ , chi-square test). Hemorrhagic signs and symptoms developed in most of the dengue fever patients in 2001; such patients were distinguished from DHF patients only on the basis that their platelet levels were  $>100,000/\text{mm}^3$ .

Of the patients with a laboratory-confirmed dengue infection and sufficient clinical and laboratory detail to confirm the grade of infection (990 patients), almost half (455) had a primary infection. However, DSS was more prevalent in patients with secondary infections (112/535) than in those with a primary infection (43/455). The median age of patients with primary infections (5 years) was not significantly different ( $p \leq 0.05$ , Wilcoxon rank sum) from those with a secondary infection (6 years). Thirty-nine of the primary infections occurred in children  $<1$  year of age; shock developed in 7 of these children. Of the remainder, 17 had DF, 12 had DHF grade I, and 3 had DHF grade II). Virus (dengue 1) was recovered from the acute-phase serum of three of these seven DSS patients. Two patients  $<1$  year of age had a secondary infection; dengue fever developed in both.

Phylogenetic analyses of the nucleotide sequences of the E protein gene of the only pre-1998 DENV-1 available from Myanmar along with 3 of the 9 isolates from 1998, both 1999 isolates, 5 of the 6 isolates from 2000, and 8 of the 115 isolates from 2001 (including an isolate recovered from a single female *A. aegypti* mosquito [My01D1m193] collected in the home of a dengue patient [My01D141500]) suggested that two new strains of DENV-1 had appeared some time before 1998, i.e., all three clades of Myanmar DENV-1 viruses have 1998 viruses in them (Figure 1). The clade containing the 1996

Table 1. Annual dengue hemorrhagic fever cases, Myanmar

Y	Cases	Y	Cases	Y	Cases	Y	Cases
1970	1,654	1978	2,029	1986	2,114	1994	11,647
1971	691	1979	4,685	1987	7,331	1995	2,218
1972	1,013	1980	2,026	1988	1,178	1996	1,854
1973	349	1981	1,524	1989	1,196	1997	4,006
1974	2,477	1982	1,706	1990	6,318	1998	12,918
1975	6,750	1983	2,756	1991	6,770	1999	5,753
1976	3,153	1984	2,273	1992	1,685	2000	1,816
1977	5,364	1985	2,666	1993	1,979	2001	15,361



Table 2. Dengue virus (DENV) serotypes recovered from patients in Yangon Children's Hospital, 1998–2001

	1998	1999	2000	2001
Clinical cases	4,596	2,342	1,100	5,616
Paired sera	709	739	501	1870
Confirmed cases	496	373	274	1,481
Sera with HI titer $\leq 80$ for virus isolation	54	30	42	236
DENV-1 isolates	9	2	6	115
DENV-2 isolates	5	5	1	1
DENV-3 isolates	9	3	1	3
DENV-4 isolates	1	1	0	0
Mixed virus isolates	0	0	0	2 (dengue 1+2)

<sup>a</sup>HI, hemagglutination inhibition

isolate (My96D123819) may be extinct (no examples have been identified since 1998). There was no apparent segregation of the viruses in the two most recent clades of Myanmar viruses according to the township (suburb) where the patient lived or to the date of onset of symptoms, i.e., viruses from both clades appeared to be co-circulating.

There were 210 nucleotide differences between the sequences of the E protein genes of the My96D123819 and My98D132514 viruses and those of the remaining Myanmar viruses. Forty-six of these resulted in amino acid changes. Amino acid changes at E37 (N-D), E155 (T-S), E161 (I-T), E329 (A-T), E369 (T-E), E442 (A-T), E468 (I-N) and E492 (T-V) distinguished these two viruses from all other Myanmar DENV-1.

The nucleotide sequences of the E genes of the viruses recovered from a patient and a female *A. aegypti* mosquito from the same house varied at three sites. Two of the changes were silent, and the third resulted in a nonconservative amino acid change at E261 from R (in the patient) to H (in the mosquito). Virus from one other patient (My00D136957) had R at this position, but virus from all other patients had the same amino acid as the mosquito at this site.

**Discussion**

The dengue outbreak in Myanmar in 2001 occurred at a time not unanticipated from the usual 3- to 4-year cycles of outbreaks in that country (Table 1). Nevertheless, we are unaware of any previous examples of dengue outbreaks, in countries in which all four dengue virus serotypes are circulating, in which a single serotype has risen to the prominence that DENV-1 appears to have reached in Myanmar in 2001.

The number of dengue cases in Yangon from 1998 to 2001 was not obviously correlated with the temperature or rainfall (Figure 2) other than the fact that the average temperature in April of the 2 epidemic years (1988, 38.5°C; 2001, 39.1°C) was almost 2°C higher than the highest average in the nonepidemic years. These observations are in broad agreement with those made in Bangkok over much larger periods (4,5) that weather was not a major factor in

determining when dengue outbreaks occurred. However, associations between meteorologic conditions and dengue outbreaks could be missed if comparisons are made between aggregated monthly totals (rainfall) or monthly averages (temperature) rather than with daily values.

Why or how the change in the composition of dengue virus populations circulating in Yangon occurred is not clear. If the DENV-1 populations diversified in the 1994–1998 interepidemic period, the changes could have been due to a genetic bottleneck, such as has been proposed to explain the disappearance of ancestral strains and

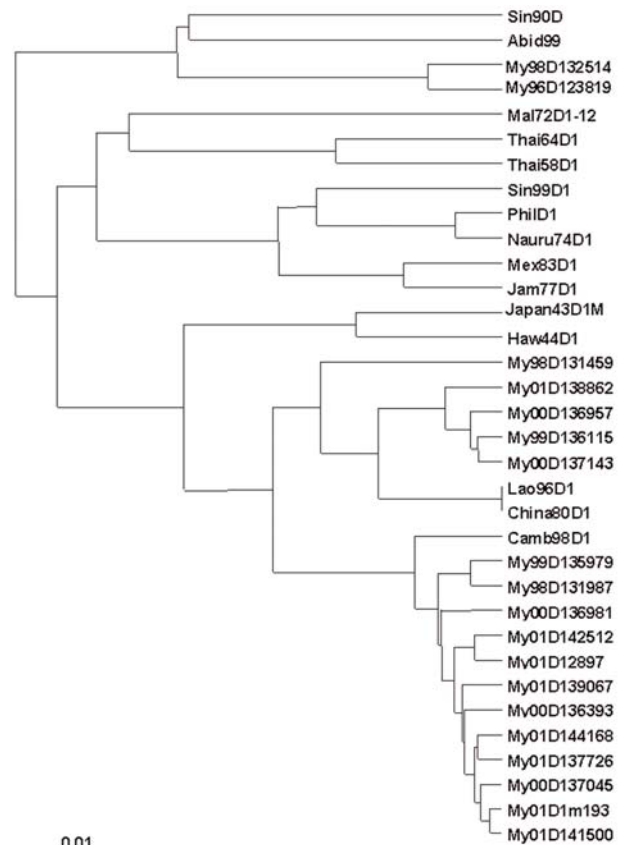


Figure 1. Phylogenetic analysis of the nucleotide sequences of the E protein genes of dengue 1 viruses from Myanmar and of dengue 1 viruses from other localities.

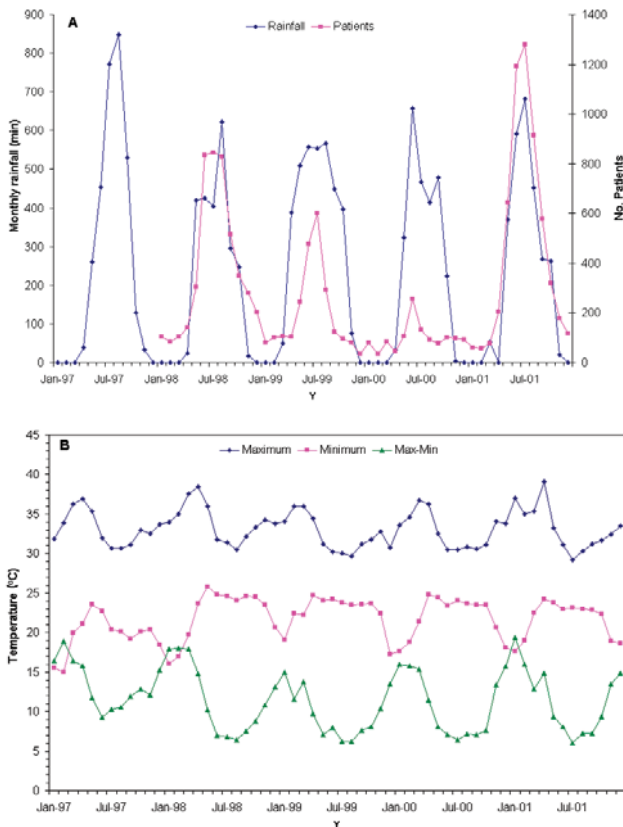


Figure 2. Relationship between weather conditions and the number of cases of dengue in Yangon, 1998–2001. (a) Comparison of the monthly admissions of dengue (dengue fever, dengue hemorrhagic fever, and dengue shock syndrome) patients to the Yangon Children's Hospital with the total monthly rainfall. (b) The average monthly maximum and minimum temperatures in Yangon from 1997 to 2001 and the difference between these values.

the appearance of new strains of DENV-2 and -3 in Thailand (16,17). Strong immunologic pressures on dengue virus populations during rapid transmission in outbreaks could give rise to serologic-escape mutant populations of virus (18). In the 1998 dengue outbreak in Yangon, in which DENV-1–4 were recovered from patients, all DENV-1 analyzed in this study were recovered in August

and September, just after the season had peaked (June 835 DHF/DSS cases; July 845 DHF/DSS cases; August 829 DHF/DSS cases; September, 516 DHF/DSS cases). Six of the eight amino acid changes (see above) that distinguished the two post-1998 DENV-1 lineages from the earlier one occurred in the portion of the E protein above the lipid membrane of the virion (E468 and E492 are in the putative transmembrane anchor region of the E protein [25]). The amino acids at E 155 of the pre-1998 lineage of DENV-1 and all 1998 strains of DENV-2–4 were the same (T) and differed from that at this position in the post-1998 DENV-1 lineages (S). At E37 and 442, the pre-1998 lineage DENV-1 isolates shared the same amino acid as 1998 lineages of DENV-2 and -3 and of DENV-2, respectively, and these differed from the amino acids in the corresponding positions of the post-1998 DENV-1 lineages (Table 3). These changes might be taken as evidence of selective pressure imposed on DENV-1 by cross-reactive antibodies produced against the co-circulating DENV-2, -3, or -4. Despite these observations, others have been unable to find any evidence of selection acting on the E protein of DENV-1 in nature (19). Furthermore, gaps in the regional virologic record make it difficult to determine whether the post-1998 strains of DENV-1 in Myanmar were introduced or whether they evolved locally.

One further difference between the observations of the dengue outbreaks in Yangon from 1984 to 1988 (7) and in 2001 was the proportion of dengue patients who had primary infections. From 1984 to 1988, 15% of patients admitted to the Yangon Children's Hospital had a primary infection (7). In 2001, 455 (46%) of 990 virologically or serologically confirmed dengue patients had a primary infection. This increase in the proportion of patients with primary infections may have contributed to the decrease in the proportion of DSS cases (6,7). Other researchers (8) have observed that primary infections with DENV-1 and -3 result in clinical disease more frequently than primary infections with DENV-2 or -4. The data from Myanmar in 2001 are compatible with these observations if the rate of isolation of each dengue virus serotype from patients in Myanmar in 2001 reflected the infection rate with each

Table 3. Comparison of amino acid changes in dengue 1 virus (DENV-1) E proteins with the sequence of the same region of co-circulating strains of DENV 2–4<sup>a</sup>

Y	Position	Amino acid sequence			
		DENV-1	DENV-2	DENV-3	DENV-4
1998	E37	MAK <b>N</b> KPT	MAK <b>N</b> KPT	MAK <b>N</b> KPT	MAQ <b>Q</b> KPT
Post-1998		MAK <b>D</b> KPT			
1998	E155	GN <b>E</b> TT <b>E</b> H	GN <b>D</b> TG <b>K</b> H	GN <b>D</b> T <b>Q</b> G <b>H</b>	GN <b>D</b> T <b>S</b> N <b>H</b>
Post-1998		GN <b>E</b> ST <b>E</b> H			
1998	E369	IEA <b>T</b> PP <b>F</b>	IEA <b>E</b> PP <b>F</b>	IEA <b>E</b> PP <b>F</b>	IE <b>L</b> EP <b>P</b> F
Post-1998		IEA <b>E</b> PP <b>F</b>			
1998	E442	IFG <b>A</b> AY <b>G</b>	VFG <b>A</b> IY <b>G</b>	DFG <b>S</b> V <b>G</b> G	VFG <b>S</b> VY <b>T</b>
Post-1998		IFG <b>T</b> AY <b>G</b>			

<sup>a</sup>Amino acids in bold occur at the positions in the E protein indicated, e.g., E37, E155.

type in the community at large. The observation of a lower incidence of DSS among patients in 2001, when most infections may have been due to DENV-1, than from 1984 to 1988 (7) or in 1998 and 1999 agrees with previous observations that DSS occurs most commonly after infections with DENV-2 in hosts who have had a prior infection with another dengue serotype (6,7). This report provides further evidence of the ability of dengue viruses to undergo rapid and unpredictable changes in genotype (16,17) and of the association of such changes with major changes in disease incidence and severity.

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# Predicting Geographic Variation in Cutaneous Leishmaniasis, Colombia

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Approximately 6,000 cases of cutaneous leishmaniasis are reported annually in Colombia, a greater than twofold increase since the 1980s. Such reports certainly underestimate true incidence, and their geographic distribution is likely biased by local health service effectiveness. We investigated how well freely available environmental data explain the distribution of cases among 1,079 municipalities. For each municipality, a unique predictive logistic regression model was derived from the association among remaining municipalities between elevation, land cover (preclassified maps derived from satellite images), or both, and the odds of at least one case being reported. Land cover had greater predictive power than elevation; using both datasets improved accuracy. Fitting separate models to different ecologic zones, reflecting transmission cycle diversity, enhanced the accuracy of predictions. We derived measures that can be directly related to disease control decisions and show how results can vary, depending on the threshold selected for predicting a disease-positive municipality. The results identify areas where disease is most likely to be underreported.

Transmission of the zoonotic disease American cutaneous leishmaniasis (ACL) is increasing in Latin America (1). ACL was originally characterized as an occupational disease of workers, primarily men, exposed to the natural transmission cycle in forests (2). Changes in these environments have led to the proliferation of various species of the sand fly vector (*Lutzomyia* spp.), their associated parasites, and reservoirs around rural settlements (3,4). In some regions, such modifications have facilitated the invasion of vectors that transmit *Leishmania* spp. associated with particularly severe disease (5). Peridomestic and domestic transmission have now been recorded in at least nine countries in the Americas (2,6) and are responsible for an increasing proportion of total cases (7,8). In the areas subject to most anthropogenic change, ACL now affects all age groups and both sexes almost equally (9,10).

The impact of ACL may be reduced by the rapid provision of antimonial drugs for treatment (11). However, the increasing incidence and domesticity of ACL also increase the feasibility of interventions to interrupt transmission around houses. To date, few control programs have been effective (12–14). Although interventions such as residual spraying of houses can reduce transmission (14,15), they are rarely applied in a focused, evidence-based manner (9). The wide geographic variation in the ecology and behavior of vectors, pathogens, reservoirs, and persons is likely to cause corresponding variation in cost-effectiveness of control measures (16). The ecologic and topographic risk factors for ACL in particular geographic regions must be clarified in order for appropriate control methods to be devised and carried out (17).

Remote sensing data are increasingly being used to measure environmental and topographic variables on the ground, and geographic information systems (GIS) are being used to model these data both spatially and temporally. The particular advantages of remote sensing include 1) numerous sensors with a wide range of spectral, spatial, and temporal resolutions (18,19), and 2) global coverage at low or no cost. These properties potentially allow GIS functions to be used to investigate environmental relationships and generate predictive maps throughout wide areas and thus focus control measures (20,21). These approaches have been used to predict distributions for a wide range of vectors and vector-borne diseases, including sand flies and leishmaniasis (22–24).

Despite their apparent utility, these techniques are still not widely used by control programs in part because health personnel often consider satellite data difficult to interpret (previous studies have usually used reflectance measurements from satellite sensors or with land-cover classifications “custom-built” by the investigators). The predictive maps that are generated are also often poorly validated and only indirectly related to control decisions. In addition, most previous analyses have been restricted to a relatively limited number of environments, vectors, or parasite species.

We used remote sensing and GIS technologies to investigate the extent to which freely available “off-the-shelf”

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(i.e., preclassified) land cover and elevation datasets can predict variation in risk for ACL transmission in Colombia, a country characterized by a highly diverse ecology, topography, and climate. These conditions have led to multiple *Leishmania* parasites (6 reported species), mammalian reservoirs (12 reported species), and *Lutzomyia* vectors (12 reported species) (15), creating a complex distribution pattern of ACL transmission (25).

We used a jackknife method previously used in ecologic studies to test the following factors: 1) the ability of statistical models based on elevation and preclassified land-cover data to predict the probability of ACL transmission in each municipality in Colombia, 2) whether predictive accuracy could be improved by allowing different environmental-disease relationships in different ecologic zones, and 3) the extent to which these predictions could also explain variation in the intensity of transmission (the reported incidence of cases) between disease-endemic municipalities. Finally, we generated various measures of model accuracy and compared their usefulness in terms of informing disease control decisions.

## Methods

### Incidence Data

Data on annual reports of ACL were obtained from the Colombian Ministry of Health. Municipality-level case reports for 1,079 municipalities in 1994 were linked to a georeferenced digitized map of municipality boundaries from the Colombian geographic institute (Instituto Agustin Codazzi) and population information from the 1993 census from the national census organization (DANE), using ESRI ArcView GIS software. This allowed municipalities with at least one reported case to be identified and incidence rates among the rural population to be calculated.

### Explanatory Data: Elevation and Land Cover

The Andean region encompasses wide variations in elevation, which is the principal determinant of variation in temperature, and strongly influences precipitation. Georeferenced elevation data from a 1-km digital elevation model of South America was downloaded from the U.S. Geological Service Earth Resources Observation System (EROS) (26). Land-cover data were obtained from the 1-km x 1-km resolution South America Seasonal Land Cover database, accessed from the same source. The data were derived from 1-km resolution, 10-day composites of NOAA-AVHRR satellite images acquired from April 1992 through March 1993, which were classified into land cover types according to their spectral characteristics throughout the year. The resulting land cover map was validated by comparing sample point pixels with cover type identified

from Landsat or SPOT images, giving an overall accuracy of 66.9% (27).

Of the 167 land-cover classes recorded in South America, 105 were represented in Colombia. However, many of these have similar or identical biologic and ecologic descriptions (Table 1), and the use of a large number of essentially replicated classes makes interpretation difficult and increases the odds of detecting an apparently significant association purely by chance. Classes with identical or highly similar descriptions were therefore grouped together. In four instances when classes were similar or identical, except for a constituent crop associated with ACL transmission (coffee), two classes were created—one with coffee and one without coffee (28–30). This process generated 25 broader classes.

Elevation and land-cover data were overlaid on the map of municipality boundaries and incidence rates, using the GIS software TNTmips (MicroImages, Inc., Lincoln, NE). This GIS was used to calculate, for each municipality, the mean elevation and the proportion of total area covered by each land-cover class.

Other mapping studies for tropical diseases (31,32) have shown that dividing predictive maps into ecologically similar areas can improve their accuracy. The ecologic, topographic, and climatic diversity of Colombia gives rise to 23 distinct vegetative zones (33) and a spatially heterogeneous

Table 1. Land-cover classification used in the analysis

Identification no.	Land-cover class label
1	Fragmented evergreen forest/grassland/savanna
2	Tropical evergreen rainforest
3	Montane evergreen rainforest
4	Submontane evergreen rainforest
5	Dry deciduous forest
6	Subtropical moist deciduous forest
7	Deciduous woodland
8	Fragmented evergreen forest/cropland
9	Deciduous forest/cropland—includes coffee
10	Fragmented evergreen forest/cropland—includes coffee
11	Cropland—includes coffee/woodland
12	Cropland—includes coffee/savanna/grassland
13	Cropland
14	Cropland/savanna/grassland/pasture
15	Cropland/ woodland
16	Fragmented montane forest/cropland
17	Grassland/savanna/woodland
18	Semiarid deciduous shrub
19	Semiarid thorn shrub/grassland/cropland
20	Flooded grassland
21	Flooded grassland/fragmented forest
22	Flooded evergreen broadleaf forest
23	Andean tundra/shrubland
24	Sparsely vegetated
25	Wooded wetland

distribution of transmission cycles and intensity of ACL transmission (15). For the purposes of this study, we follow Espinal and Montenegro in dividing Colombia into seven ecoregions (33). Of these, two small regions were either combined with larger contiguous regions (Catumbo River Basin joined to Magdalena River Valley) or excluded from the analysis (Central Andean Massif). This left five zones for ecoepidemiologic analysis: Pacific, Atlantic, Amazon and Eastern Plains, Cauca River Valley, and Magdalena River Valley. These ecoregions, and the geographic distribution of elevation and vegetation are illustrated in Figure 1.

### Statistical Analysis of Ecologic Associations

Predictions of the probability of transmission were generated by using a jackknife procedure (34). In this approach, a single municipality was excluded, and a logistic regression model was fitted to the remaining data. The response variable was defined as either presence or absence of at least one reported case of ACL, and the independent variables were defined as the proportions of the total area of each municipality belonging to each land-cover class, mean elevation, and (to allow nonlinear relationships) the square of the mean elevation. The coefficients from this model were then applied to the values of the predictor variables from the missing municipality to generate a predicted probability of occurrence between 0 and 1. The process was repeated for each municipality. Predicted and observed datasets were therefore independent because the prediction for each municipality was generated by using disease data only from other locations. The statistical significance of the fit was measured by using the chi-square value from a logistic regression of the observed data against the predicted data for all municipalities.

We compared the predictive power of different types of explanatory data using 1) both land cover and elevation

information, 2) only elevation information, and 3) only land cover information. To measure the value of dividing the study area into more ecologically homogenous regions, each of the regression procedures was then repeated, but predictions for each municipality were generated by using only data from the same ecologic zone. To assess similarity in ecologic relationships between regions, predictive models from each zone were also used to predict occurrence in all other zones. As the zones are independent, a complete model that included all data from one zone was used to predict the presence or absence in the other zones.

Predictions of presence or absence are often assessed by comparing predictions and observations to measure sensitivity (ability to correctly predict “true” positives), specificity (ability to predict true negatives), positive predictive value (PPV; proportion of predicted positives that are truly positive), negative predictive value (NPV; proportion of predicted negatives that are truly negative) (35), and  $\kappa$  statistics (the proportion of observations that we would have expected to be incorrectly predicted on the basis of chance, but which are correctly predicted, i.e., a measure of the additional “skill” of the model over chance).

Because the above procedure predicts a probability of transmission between 0 and 1, a threshold probability must be selected to convert these values into predictions of presence or absence. This selection influences the value of all of the above measures, but this choice is arbitrary unless other factors must be considered, such as differential costs and benefits of identifying positive versus negative locations. We therefore followed a procedure previously used in ecologic and veterinary mapping studies (36–38), and more recently applied in human disease mapping (32), of plotting sensitivity against (1-specificity) for all thresholds between 0 and 1, to generate a receiver-operator curve. The area under the receiver-operator curve (AUC) gives a single comparable measure of overall model performance,

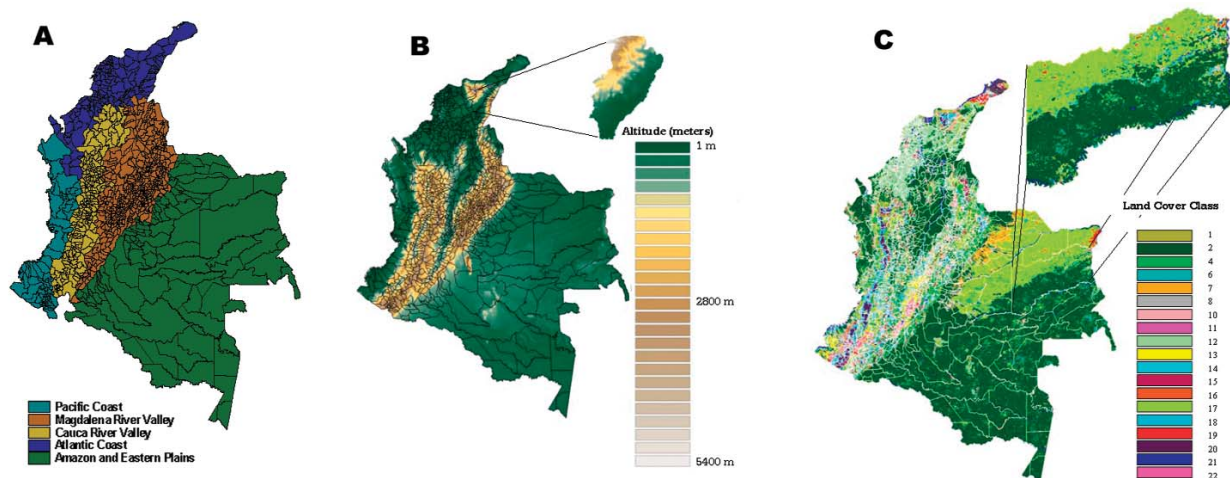


Figure 1. Distribution of A) ecoepidemiologic zones, B) elevation, and C) vegetation types in Colombia.

reflecting the proportion of occasions on which a randomly selected location with transmission has a predicted probability greater than that for a randomly selected location without transmission. We also calculate sensitivity, specificity, PPV, NPV, and kappa across all thresholds, for both the single model and the combination of the zonal models. Finally, we assessed the ability of the model to predict variation in incidence between disease-endemic municipalities by regressing the log-transformed values for incidence against predictions of probability of transmission, using values from the model that performed best in the above tests.

## Results

### Epidemiologic Data

The reported incidence of ACL (undoubtedly an underestimate of true rates) has more than doubled from the early 1980s to the late 1990s (Figure 2). Table 2 summarizes reported ACL incidence in 1994, and the main parasite and vector species, in each ecoepidemiologic region. Despite very different ecologic characteristics and transmission cycles, each zone has an approximately equal proportion of municipalities reporting ACL transmission.

Figure 3 shows the geographic distribution of reported ACL incidence, by municipality. Transmission is absent from the highest elevations along the eastern and western cordilleras of the Andes, presumably because of low temperatures. Elsewhere, transmission is highly heterogeneous, with a small proportion of municipalities reporting a high proportion of the total cases. For example, 50% of the reported cases were from only 20 (1.9%) of all municipalities.

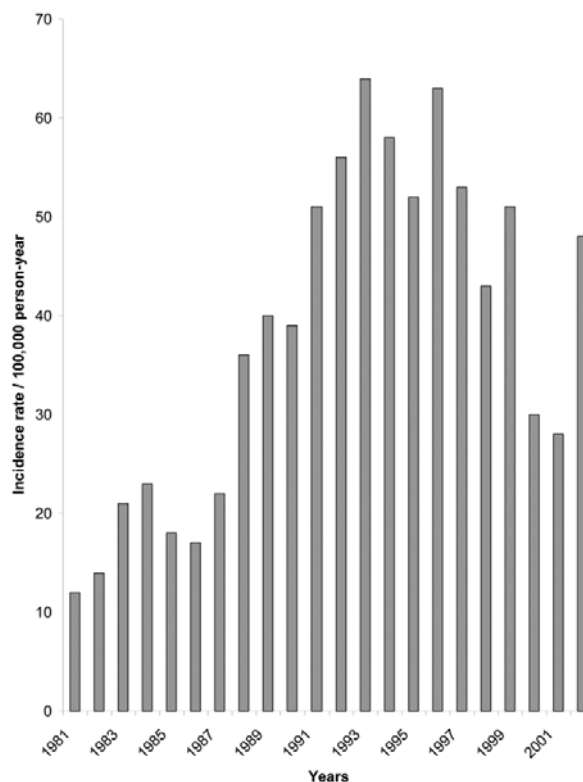


Figure 2. Incidence of American cutaneous leishmaniasis per rural population reported in Colombia by year, 1980–2002 (data from Ministerio de Salud, Colombia).

### Environmental Predictions of ACL Transmission

Table 3 summarizes the performance of logistic regression models in predicting the geographic distribution of municipalities with at least one reported case of ACL. Perhaps the best measure of overall model performance is

Table 2. Reported incidence of ACL, Colombia, 1994, and major parasite and vector species, by ecoepidemiologic region<sup>a</sup>

Region	Total municipalities	Positive municipalities (% positive)	Median, range of incidence in positive municipalities (/100,000 rural pop.)	Principal vectors	Principal parasite species
Amazon and Eastern Plains	105	42 (40)	62 (7–1,448)	<i>Leishmania carrerai</i> , <i>L. umbratilis</i>	<i>L. amazonensis</i> , <i>L. braziliensis</i> , <i>L. guyanensis</i> , <i>L. mexicana</i> , <i>L. panamensis</i>
Atlantic	152	50 (33)	57 (2–3,030)	<i>L. ovallesi</i>	<i>L. panamensis</i>
Cauca River Valley	248	56 (23)	29.5 (3–944)	<i>L. colombiana</i> , <i>L. trapidoi</i> , <i>L. youngi</i>	<i>L. braziliensis</i> , <i>L. panamensis</i>
Magdalena River Valley	496	136 (27)	64.5 (4–6,662)	<i>L. gomezi</i> , <i>L. hartmani</i> , <i>L. longiflocosa</i> , <i>L. ovallesi</i> , <i>L. panamensis</i> , <i>L. spinicrassa</i> , <i>L. torvida</i>	<i>L. braziliensis</i> , <i>L. panamensis</i>
Pacific	77	25 (32)	117 (6–1,789)	<i>L. gomezi</i> , <i>L. trapidoi</i>	<i>L. braziliensis</i> , <i>L. mexicana</i> , <i>L. panamensis</i>

<sup>a</sup>ACL, American cutaneous leishmaniasis; pop., population.

Table 3. Diagnostic statistics of predictive models for presence/absence of ACL transmission

Type of model	Predictors used	Accuracy measures <sup>b</sup>					
		AUC	Maximum $\kappa$	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Single model for whole country	Elevation	0.66	0.23	59.9	65.8	41.3	80.3
	Land cover	0.70	0.28	53.4	75.7	46.9	80.2
	All	0.72	0.34	55.3	79.3	51.8	81.6
Combination of zonal models	Elevation	0.70	0.28	53.7	75.2	46.5	80.2
	Land cover	0.82	0.46	67.0	80.6	58.1	85.9
	All	0.84	0.54	62.8	89.1	69.8	85.6

<sup>a</sup>ACL, American cutaneous leishmaniasis; AUC, area under receiver-operator curve; PPV, positive predictive value; NPV, negative predictive value. Sensitivity, specificity, PPV, and NPV are calculated at the probability threshold that gives the highest value of kappa.

<sup>b</sup>For all comparisons of observations against predictions,  $\chi^2 > 79.2$ ,  $df = 1$ ,  $p < 0.0001$ .  $\kappa$  values are given by  $(\text{proportion correct} - \text{Proportion expected}) / (1 - \text{proportion expected})$ , where proportion correct =  $(a + d)/n$ , and proportion expected =  $(a + b) \times (a + c) + (c + d) \times (b + d) / n^2$ . a = true positive predictions, b = false positive, c = false negative, d = true negative, n = total.

the area under the receiver-operator curve. As a guide, values from 0.5 to 0.7 indicate a poor discriminative capacity, 0.7–0.9 indicate reasonable capacity, and  $>0.9$  indicate a very good capacity. A value of 0.5 is expected by chance (39).  $\kappa$  values vary with threshold, so only the maximum  $\kappa$  value is quoted here.  $\kappa$  values below 0.4 can be considered to show poor agreement; 0.4–0.75, good agreement; and above 0.75, excellent agreement (40). Values for the other properties also depend on choice of probability threshold, and those shown here correspond to the threshold that gives the highest  $\kappa$  (i.e., where the model has the greatest additional predictive power, above that expected by chance alone).

Each modeling approach gives predictions that are significantly better than chance. However, predictions based on zonal division of the country are markedly more accurate than those from a single analysis, demonstrating the advantage of allowing the model to describe different relationships between environment and disease in different ecologic regions. Within either single or zonal modeling approaches, land-cover information from the preclassified satellite images gives greater predictive power than elevation information alone. The most accurate predictions are given by combining both elevation and land cover information.

Figure 4 compares the accuracy of predictions from the single model and the combination of the zonal models. Figure 4A shows the receiver-operator curve. The improved overall performance of the zonal model is indicated by the curve more closely approaching the top left corner, which represents both maximum sensitivity and specificity. Figure 4B shows how the  $\kappa$  statistic varies with choice of threshold, and indicates that both models have greater skill at intermediate probabilities, but that the zonal model has greater skill over a wider range of probability thresholds.

Figure 5 uses the predictions from the zonal model to illustrate the effect that the choice of probability threshold has on measures of model accuracy. Figure 5A shows the clear trade-off between sensitivity and specificity: sensitivity is maximized by selecting low threshold values and

specificity by selecting high threshold values. Similarly, Figure 5B shows that PPV tends to be higher at greater threshold values, and NPV tends to be higher at lower thresholds, but that the relationship is nonlinear. No single probability threshold optimizes all desirable properties of the predictive model.

Table 4 shows the ability of models generated using data from a single region to predict transmission within the same zone or other zones. The high AUC values on the diagonal confirm that the models are accurate in predicting transmission within the same region. In comparison, they are much less able to predict for ecologically dissimilar zones. Models based on the larger regions (Cauca and

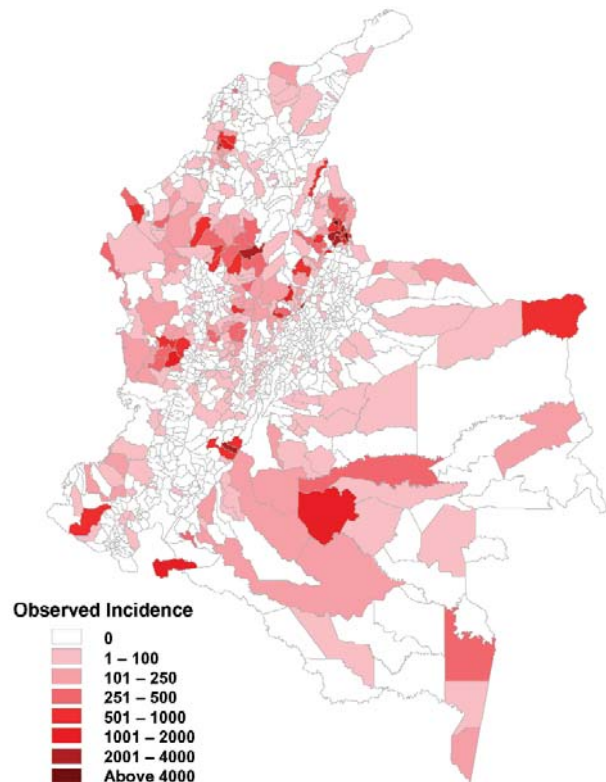


Figure 3. Geographic distribution of American cutaneous leishmaniasis incidence by municipality, 1994



Magdalena River Valley) have moderate predictive value for other areas, while those based on smaller regions tend to have poor predictive value and may generate worse predictions than those expected by chance (AUC <50%).

### Generation of Risk Maps and Comparison with Observed Data

The predictions from the zonal model were used to generate a risk map for probability of transmission occurring in each municipality (Figure 6). The results shows a good match with the observed distribution of transmission (Figure 3). Figure 7 directly compares the two maps. Municipalities were predicted as ACL-endemic or ACL-nonendemic, using the combination of the zonal predictions and applying the probability threshold that gives the maximum value of  $\kappa$ . False-negative predictions (i.e., municipalities where transmission was reported but not predicted by the model) presumably reflect poor ability of the model to describe the effect of local environmental characteristics on transmission risk. False-positive predictions (i.e., transmission was predicted but not reported) may also indicate poor model fit, or alternatively, areas where transmission is occurring but has not been reported.

### Predictions of Variation in Incidence Rates

Regression of log-transformed incidence data in positive municipalities against predicted probability of disease transmission showed a highly significant positive correlation ( $\ln[\text{incidence}] = 3.43 + 1.51 \times \text{predicted probability}$ ,  $F_{1,305} = 19.04$ ,  $p < 0.001$ ), indicating that the models also have some value in predicting transmission intensity. However, the regression explained only a small proportion of the variance (6%), and did not describe the true range of variation in incidence, whereas the fitted line gave predictions of log incidence between 3.43 and 4.94 (corresponding to incidence rates of 31 to 140 per 100,000 rural population); observed incidence rates ranged between 2 and 6,662 per 100,000.

### Discussion

During the last decade or so, multiple studies have shown the utility of remote sensing and GIS analysis for identifying the ecologic determinants of the distributions of parasitic diseases, and thereby generating predictive maps. For disease control programs to apply and act on these techniques, however, the resulting predictive maps should 1) give accurate predictions against independent data, 2) be based on predictor data that are inexpensive and easy to interpret, and 3) generate outputs that are directly related to control decisions.

The modeling and validation procedure used here has not previously been used in mapping vector-borne disease. However, previous ecologic applications highlight several

advantages over alternative methods, such as using the full dataset for both model development and validation (which overestimates predictive accuracy), or dividing the dataset into independent "training" and validation sets (which involves a subjective decision over division of data, and a reduction in sample size) (34). Each prediction generated here is based on the maximum sample of independent data and reflects the process of attempting to predict the next observation in an accurate and unbiased manner.

Application of this technique shows that readily available land cover maps, preclassified from NOAA-AVHRR satellite data, can help accurately predict the presence or absence of ACL transmission at the municipality level across a large, ecologically and geographically diverse country. These maps have a substantial advantage in that

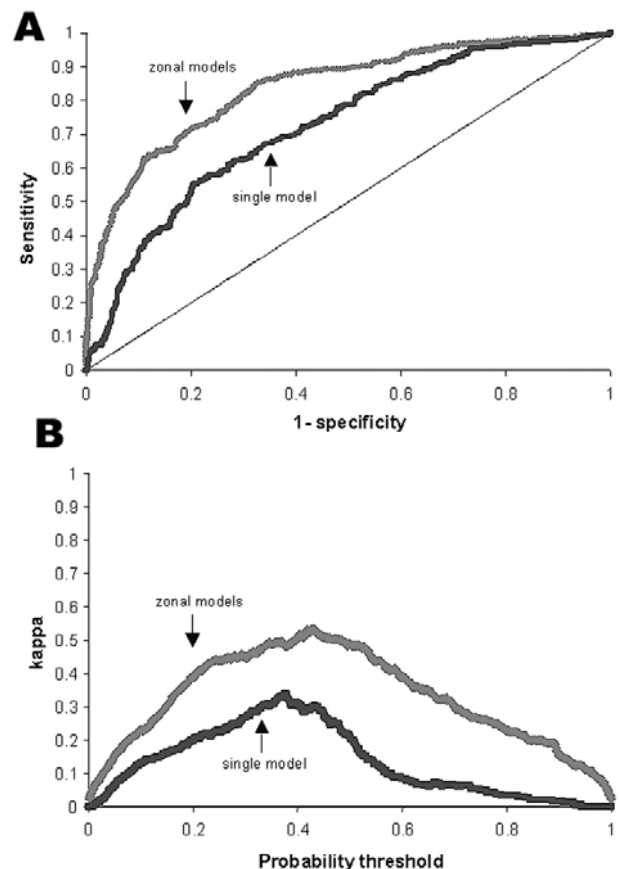


Figure 4. Performance of whole country model versus combination of zonal models. A. Receiver operator curve. Black line, single model for all Colombia (area under the curve [AUC] = 72.4%); gray line, combination of zonal models (AUC = 84.4%). Diagonal line indicates success expected on the basis of chance (AUC = 50%). B.  $\kappa$  value, representing skill at discriminating positive and negative municipalities, above the level expected on the basis of chance. Black line, single model for all Colombia; gray line, combination of zonal models. The probability threshold is the value on the continuous scale of predicted probability of transmission that is used as the cut-off for conversion into a categorical prediction of presence versus absence.

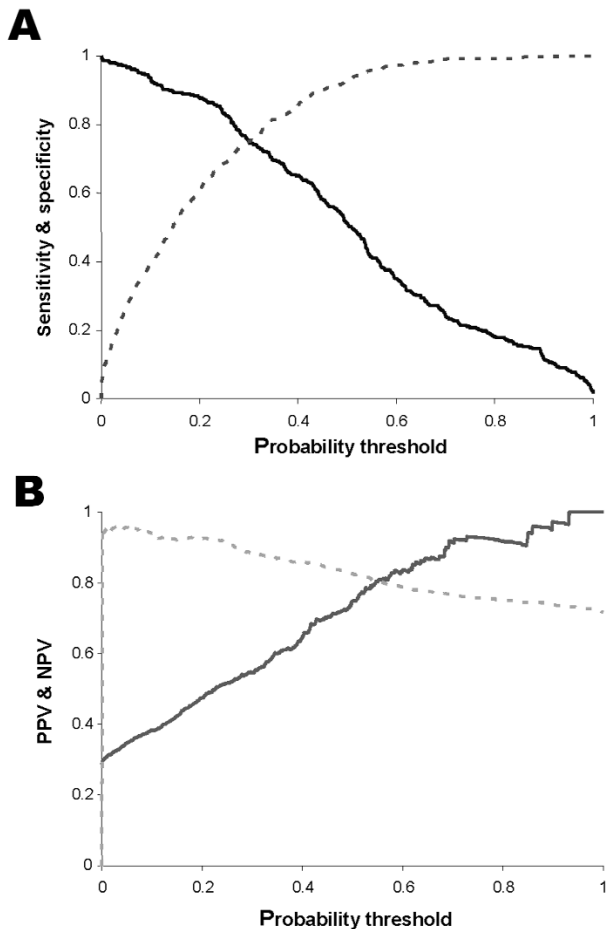


Figure 5. A. Sensitivity (solid line) and specificity (broken line) across the range of threshold probabilities for predicting an endemic municipality. B. Positive predictive value (solid line) and negative predictive value (broken line) across the range of threshold probabilities for predicting a positive municipality. The probability threshold is the value on the continuous scale of predicted probability of transmission that is used as the cut-off for conversion into a categorical prediction of presence versus absence.

they can be easily manipulated and interpreted by persons with basic GIS skills, ecologic knowledge, and computer equipment, without requiring detailed technical knowledge of the properties of satellite sensors or the reflectance properties of different land-cover types.

As in other mapping studies of ecologically diverse areas (31,32), the accuracy of model predictions is improved when different statistical models are applied in distinct areas. Although the zones used here are defined by using general ecologic characteristics, future models could potentially be further improved by defining zones based specifically on the distribution of the principal sand fly vectors.

The remaining prediction errors are likely due to several factors. These include the spatial resolution of the

AVHRR data and accuracy of classification into land cover types, the procedure for grouping into larger classes, and the slight difference between dates of collection of satellite and disease data. The factors also include nonecologic explanatory variables not captured in the model, such as variation in human behavior and housing quality. Alternatively, the errors may reflect limitations in the surveillance system, such as unreliable diagnosis and notification, or cases acquired in municipalities other than where they were diagnosed and reported. The models generated here are relatively poor at predicting the variation in transmission intensity within positive municipalities. The reasons for this are unclear; the characteristics captured in our models may be useful in defining the minimum ecologic conditions necessary for transmission, but other influences such as the demographics, behavior, and herd immunity of human and reservoir populations may exert a stronger influence on incidence rates within these ecologically suitable areas.

The techniques used here have two main practical applications. First, they allow simple hypotheses about the major determinants of disease distributions to be tested. For a country with a large range in altitude, elevation has relatively poor predictive power compared to land cover. Alternative elevation measures (such as minimum elevation, terrain roughness, average slope) might provide more explanatory value. Because land cover is partly determined by elevation, the various land-cover classes described here do effectively integrate elevation data with other ecologic influences, such as latitude, rainfall, and human influences on the environment. Nevertheless, both types of data improve predictive accuracy, and merit collection and application in risk mapping.

The use of preclassified land-cover data facilitates biologic interpretation and suggests what may happen with specific future land use changes (e.g., the replacement of forest with common crops). The analysis could be further developed by updating with more recent land-cover information, by applying species-specific information on the relationship between vectors and vegetation in order to refine the grouping of land-cover classes, and by investigating whether particular land-cover types are associated not only with incidence but also with the level of peridomestic transmission, as indicated by relative infection rates in children versus adults, or by the abundance of sand fly vectors with known domestic behavior. Our approach can also serve as a “first-cut” to identify areas of particular interest (either high transmission risk or with specific ecologic associations) that could be further investigated by using satellite imagery with greater spatial resolution (19).

The second practical application of these datasets and analyses is for generating predictive maps that can be used

Table 4. Accuracy measurement (area under the curve) for models generated using data from one region, assessed within the same region, and in other regions

Assessment region	Model region				
	Amazon and Eastern Plains (%)	Atlantic (%)	Cauca River Valley (%)	Magdalena River Valley (%)	Pacific (%)
Amazon and Eastern Plains	83.4 <sup>a</sup>	46.6	52.2	54.7	52.2
Atlantic	45.8	85.2 <sup>a</sup>	57.9	56.1	48.2
Cauca River Valley	56.3	51.7	82.2 <sup>a</sup>	60.2 <sup>b</sup>	50.3
Magdalena River Valley	66.2 <sup>a</sup>	56.7	67.7 <sup>a</sup>	82.7 <sup>a</sup>	52.2
Pacific	59.2	54.5	67.0 <sup>c</sup>	70.9 <sup>b</sup>	93.6 <sup>a</sup>

<sup>a</sup>p < 0.0001, for fit of predictions against observations.  
<sup>b</sup>p < 0.01  
<sup>c</sup>p < 0.05

to target resources (e.g., drugs or insecticide spraying activity) between municipalities when notification data are incomplete or unreliable. No risk map is 100% accurate, and a range of statistics are available for assessing model quality. AUC is an appropriate measure for comparing the overall performance of different models. However, comparisons should ideally be made in relation to a specific control decision and with a priori knowledge of the particular characteristics that the control program is attempting to optimize. For example, sensitivity indicates the probability of correctly identifying a disease-endemic municipality. If sensitivity is low, many communities at high risk will not receive the resources they require. In contrast,

PPV measures the probability that a community, which we predict to have high risk, is truly at high risk. If PPV is low, a risk map could lead to a waste of resources and unnecessary environmental or health damage as the result of insecticide spraying. Therefore, the final assessment of the utility of a risk map requires a full analysis of the relative economic costs and health benefits of decisions made based on its predictions.

An apparent paradox exists in that predictive mapping attempts to estimate disease risk in areas with poor or missing data, yet all mapping studies ultimately rely on the quality of the underlying data. More and higher quality data

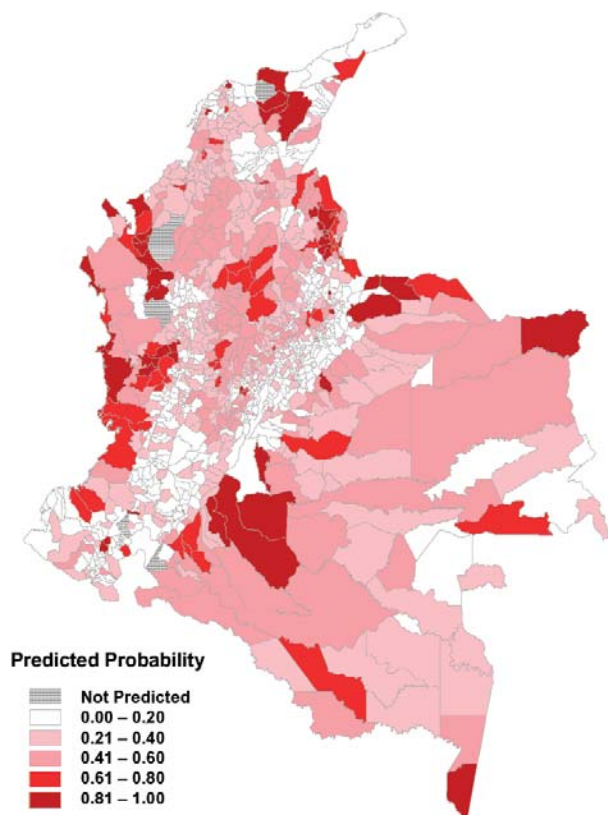


Figure 6. Predicted risk map for probability of transmission, based on the combination of the regional models.

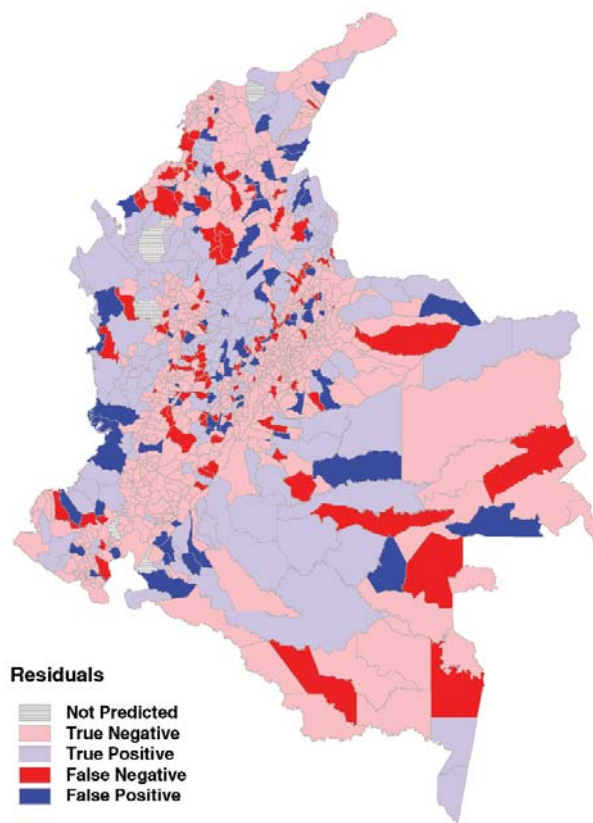


Figure 7. Agreement between predictions and observations. Light blue, correct positive prediction; light red, correct negative; dark blue, false positive; dark red, false negative.

should lead to improved hypothesis testing and predictions, yet as the quantity and quality of the reporting data improve, predictions are needed less. Predictive modeling and data collection may therefore be most productive when used as complementary iterative processes, with models highlighting where new data collection is most important, and surveillance data are continually improving the models. In this process, errors are more informative than correct predictions. In our example, prediction errors indicate either sites where transmission occurs but is not predicted (suggesting different ecologic relationships requiring further research) or sites where transmission is predicted but not observed (possibly representing under-reporting, and therefore priority areas when revising surveillance systems). Predictive mapping is not a replacement for ecologic fieldwork and reliable reporting systems but can be a useful tool for directing each of these fundamental activities and extracting maximum value from them.

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# Epidemiologic Determinants for Modeling Pneumonic Plague Outbreaks

Raymond Gani\* and Steve Leach\*

Pneumonic plague poses a potentially increasing risk to humans in plague nonendemic regions either as a consequence of an aerosolized release or through importation of the disease. Pneumonic plague is person-to-person transmissible. We provide a quantitative assessment of transmissibility based on past outbreaks that shows that the average number of secondary cases per primary case ( $R_0$ ) was 1.3 (variance = 3.1), assuming a geometric probability distribution, prior to outbreak control measures. We also show that the latent and infectious periods can be approximated by using lognormal distributions with means (SD) of 4.3 (1.8) and 2.5 (1.2) days. Based on this parameter estimation, we construct a Markov-chain epidemic model to demonstrate the potential impact of delays in implementing outbreak control measures and increasing numbers of index cases on the incidence of cases in simulated outbreaks.

*Yersinia pestis* causes an enzootic vector-borne disease infecting rodents and fleas; humans can also become infected when exposed to zoonotic reservoirs. Infection in humans usually occurs in the form of bubonic plague when fleas that have previously fed on plague-infected rodents bite them. Secondary pneumonic plague may then occur if infection spreads to the lungs. Persons with secondary pneumonic plague become infectious and can transmit the disease to other persons by the respiratory route, causing primary pneumonic plague (1,2). Primary pneumonic plague is also person-to-person transmissible and can sustain cycles of human transmission independent of flea and rodent vectors. Bubonic plague can usually be treated successfully with antimicrobials; however, secondary pneumonic plague and primary pneumonic plague require prompt antimicrobial treatment. Symptoms develop rapidly and are usually fatal (1,3,4). The recent discovery of antibiotic-resistant strains of *Y. pestis* (5) poses potential new concerns for therapeutic and prophylactic treatments during outbreaks.

The risk of importing *Y. pestis* to nonendemic regions may have increased over recent years. The worldwide extent of plague endemic-areas and the global incidence of reported disease have both increased (6), as have the volume and rapidity of national and international trade and travel. These factors raise the likelihood of importation either through travelers incubating plague (as occurred in New York 2002 [7]), or through importation of infected vectors, such as fleas or rats. Imported vectors then have the potential to initiate outbreaks of pneumonic plague.

Plague is also recognized as a potential weapon for bioterrorists (3,8–11) and has been used, or considered for use, as a biologic weapon in the past. From the 14th to the 18th century in Europe, attempts were made to spread plague in besieged cities by catapulting plague victims over the walls (12). During the 1930s, the Japanese military attempted to spread plague in China by dropping plague-infected fleas from aircraft (12). As late as the 1990s, the Union of Soviet Socialist Republics was developing plague as an aerosol agent to cause primary pneumonic plague in target populations (9). Recent training exercises in the United States have been conducted to test the abilities of healthcare systems to cope with large-scale aerosolized releases of *Y. pestis* into urban populations (13,14).

Given that primary pneumonic plague is transmissible person-to-person and outbreaks could occur as a consequence of importation or bioterrorism, it is essential to develop quantitative assessments of the transmissibility and kinetics of the disease that are as robust as possible to aid public health planning, including training exercises such as those referred to above. Without preparation, inappropriate responses such as those seen during the suspected outbreak of plague in Surat, India (1994), are inevitable; the tourist industry suffered, exports were affected, and excessive demands were placed upon healthcare systems. The losses in this case have been estimated to run into billions of U.S. dollars (15).

While there has been much discussion concerning the transmissibility of primary pneumonic plague, no quantitative estimates could be found in published literature. The

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qualitative assessments that were found varied considerably: some reports suggest that primary pneumonic plague is highly transmissible and infectious (1,16–19), while others suggest that it is not (20,21) or that intimate contact between persons is required for transmission (22,23).

Using mathematical models based on historic data, we quantitatively assess the transmissibility and potential health effects of primary pneumonic plague outbreaks under a range of assumptions. In this initial analysis, we consider only the immediate health effects due to primary pneumonic plague and not the possible long-term effects due to potentially establishing the pathogen in rodent reservoirs and subsequent risks for bubonic plague. Based on available epidemiologic evidence, the modeling assumes that persons, once infected, experience a non-symptomatic latent period followed by a symptomatic infectious period during which they can transmit primary pneumonic plague to other persons. Thereafter, if infected persons are untreated they will die. The reported case-fatality rate is close to 100% (1,3,4).

To estimate the duration of the latent period and the infectious period, and the probability of transmission of primary pneumonic plague, data describing cases and transmission events were sought from well-documented outbreaks. Reports of sufficiently well-documented outbreaks were rare, and each of the outbreaks resulted in relatively small numbers of new cases of primary pneumonic plague. Since therapy may affect the duration of individual latent periods and infectious periods, only the data in reports from person who had not received therapy was used in this analysis for latent periods (24–29), and for infectious periods (24,25,27,28). Lognormal distributions were fitted to these data by maximizing the log-likelihood function. In subsequent modeling, the duration of individual latent periods and infectious periods could then be taken from the fitted lognormal distributions in Figure 1 with means (SD) of 4.3 (1.8) and 2.5 (1.2) days.

To estimate the transmission rate of primary pneumonic plague, only those transmission events from reports where the infecting persons could be unambiguously identified and where the infections had occurred before public

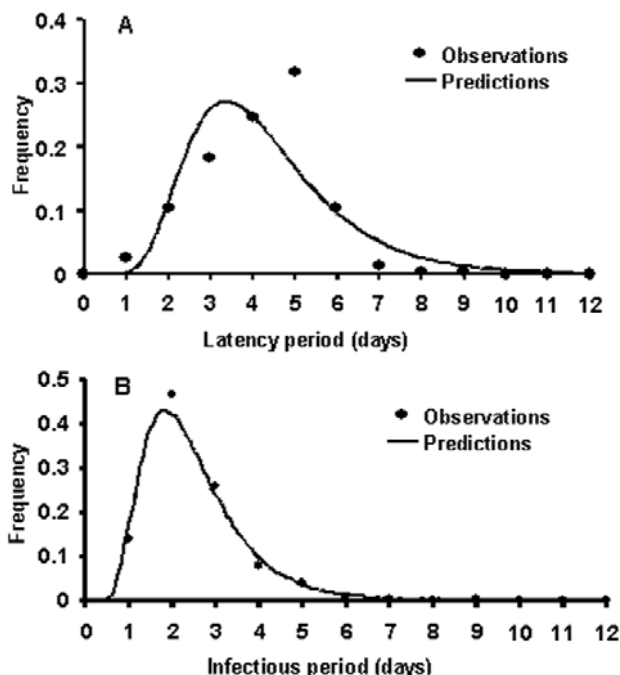


Figure 1A. Frequency distribution for the latent period with a fitted lognormal distribution ( $n = 224$ ); B. frequency distribution of the length of the infectious period with a fitted lognormal distribution ( $n = 225$ ).

health intervention were included in the analysis. The average number of infections generated by each infected person was then determined for each of the outbreaks documented in the Table, which varied from 0.8 to 3.0 (this variation most likely reflects the stochasticity that is inherent in very small outbreaks—see also discussion below). To obtain a stronger and more generalized estimate of transmissibility across all of the outbreaks, probability density functions (e.g., Poisson, geometric), were fitted to these data by maximizing the log-likelihood function for the probability and frequency of individual transmission events aggregated across the datasets. The geometric distribution gave the best fit to the data ( $f(x) = p(1-p)^x$ , where  $x =$  no. secondary cases per primary case,  $f(x) =$  frequency and  $p = 0.43$ ), and predicted an average of 1.3 secondary

Table. Documented outbreaks of primary pneumonic plague (PP) from which transmission data were derived

Y and location	Total of PP cases in outbreak <sup>a</sup>	No. of PP cases before intervention <sup>b</sup>	Transmission events prior to interventions	Average no. of secondary transmissions per primary transmission
Seattle, USA, 1907 (30)	5	5	4	0.8
Oakland, USA, 1919 (24)	13	6	12	2.0
Ecuador, 1939 (23)	18	4	6	1.5
Mukden, China, 1946 (25)	39	9	8	0.9
Rangoon, 1946 (31)	16	11	22	2.0
NW Madagascar, 1957 (32)	42	35	39	1.1
Zambia, 1993 (33)	3	3	2	0.7
Madagascar, 1997 (26)	18	1	3	3.0

<sup>a</sup>Includes index case.

<sup>b</sup>Only includes cases in which the infecting person could be identified.

cases per primary case ( $R_0$ ) with variance of 3.1. This provides a probability density function (Figure 2) which was used in subsequent modeling to calculate the expected number of secondary cases per primary case for each person infected with primary pneumonic plague.

Documented 20th century outbreaks of primary pneumonic plague were often rapidly contained once they came to the attention of public health authorities (Figure 3). Even in the pre-antimicrobial era when outbreaks were not specifically identified as plague (e.g., the outbreak in Oakland in 1919 [24] that was thought to be a deadly form of influenza), the isolation of ill persons and observation and isolation of contacts were sufficient to rapidly control the outbreak. Contact tracing and isolation tended to be immediately effective because patients were infectious for only a short time, were very ill and unlikely to go out into

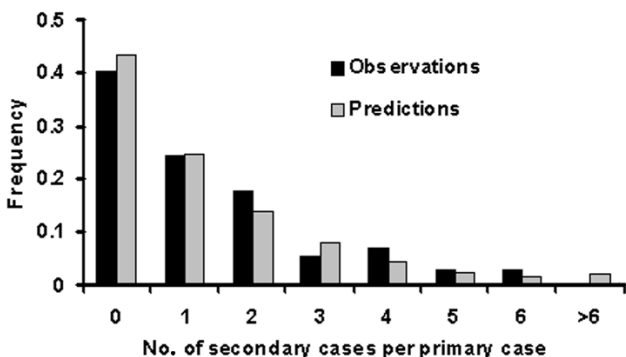


Figure 2. Frequency distributions for the number of secondary cases per primary case of primary pneumonic plague. Observations from outbreaks in Table are in black and the fitted geometric distribution in gray.

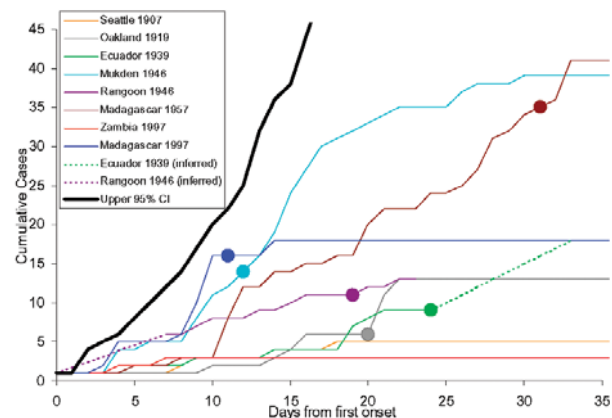


Figure 3. Epidemic curves for outbreaks in Table and from the model. The curves plot cumulative cases at time of onset. Day 0 is the time of onset of index case, the circles represent the times at which disease control measures begin, those without circles ended without public health interventions. Dotted lines indicate missing data. The thicker black line represents the upper 95th percentile from the epidemic model, which rises roughly exponentially to a value of 256 by day 35.

the community, and any subsequent infections tended to be in those already caring for the patient (Figure 4). Very rarely were there cases where a prior infectious contact could not be identified. In addition, modern antimicrobial prophylaxis, when given in the incubation period, is close to 100% effective for pneumonic plague, greatly reducing any prospects of transmission from infected, but not yet symptomatic, persons (3,22,26,34,35). The subsequent modeling therefore assumes that once an outbreak has been identified, further transmission will be stopped. It is further assumed that a cumulative number of deaths are likely to have occurred before an outbreak comes to the attention of public health authorities and appropriate interventions are put in place, denoted  $D_0$ .

A simple Markov-chain model was used to model disease outbreaks such that an individual  $i$  would have a latent period of  $L_i$  and an infectious period of  $I_i$ , where  $L_i$  and  $I_i$  were random deviates selected from the appropriate probability density functions in Figure 1. The individual  $i$  would then infect  $T_i$  susceptible persons, where  $T_i$  was a random deviate selected from the geometric probability density function described in Figure 2. As a simplifying assumption, new infections were assumed to occur within 1 day of  $i$  becoming infectious, as new infections were usually in close personal caregivers, few in number, and the symptomatic period of short duration. The upper 95th

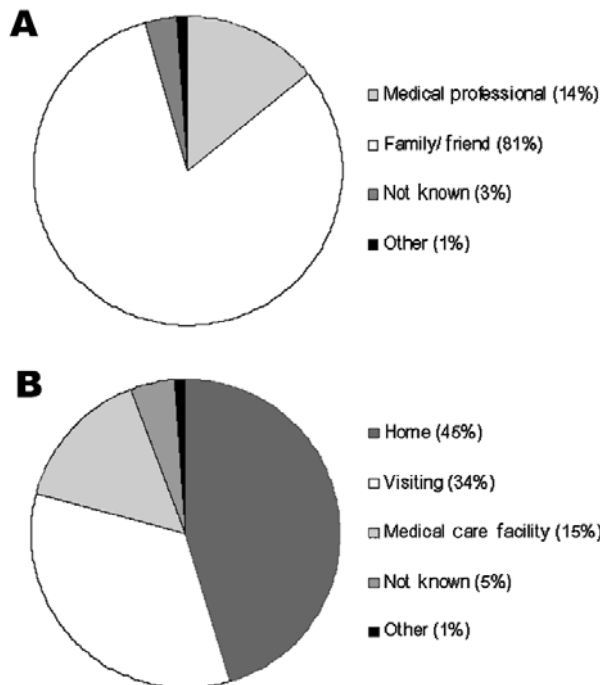


Figure 4. Distributions for the contexts of the transmission events for PPP by (A) type of contact with infectious individual ( $n = 91$ ), and (B) location of infectious contact when infected ( $n = 86$ ). Data aggregated from multiple sources (23–26,30–33), where these data were specified).



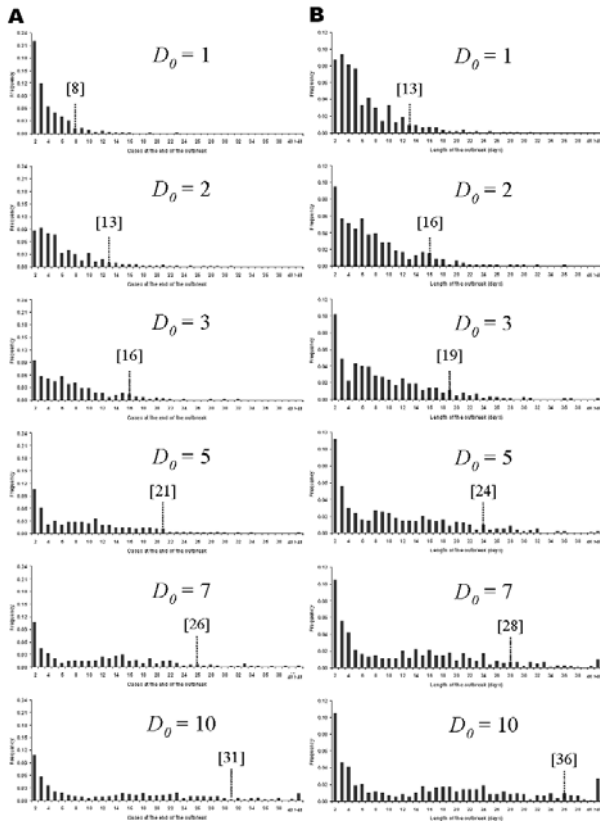


Figure 5. Frequency distributions for (A) the expected number of cases at the end of outbreaks, and (B) the expected lengths of outbreaks when different numbers of deaths are required to trigger public health interventions. The values in the square brackets refer to the value at the upper 95th percentile value. For a larger reproduction of this figure, please see [www.cdc.gov/ncidod/EID/vol10no4/03-0509-G5.htm](http://www.cdc.gov/ncidod/EID/vol10no4/03-0509-G5.htm)

percentile from the multiple iterations of the model with no interventions applied is shown in Figure 3, along with the epidemic curves for each of the outbreaks listed in the Table. From the timings of the public health interventions that are shown in Figure 3, it is clear, with the exception of Mukden, 1946 (25), that the control measures were very effective in controlling all outbreaks; any subsequent cases occurred only as a result of infections incurred before the initiation of the control measures.

After the introduction of latent infections into a community, infectious symptomatic cases will begin to appear over time. By the time an outbreak has been detected, there will potentially be a number of infectious persons in the community that can be estimated by using the modeling procedure described above. This number is critical in estimating the likely scale of response that might be required by public health authorities, giving a guide not only to the number of infectious people in the community at that point, but also an index for further onward transmission

should responses be delayed. The model was thus used to numerically estimate a function, given by equation 1, that estimates the average number of infectious persons in the community with the potential to infect others,  $I(t)$ , at different times,  $t$ , following the initial introduction of different numbers of infections ( $N_0$ ) into the population and prior to control measures being applied (i.e., prior to  $D_0$  deaths having occurred).

$$I(t) = \alpha N_0 e^{\beta t} \text{ (equation 1)}$$

where  $\alpha = 0.3841$  (SE = 0.00078) and  $\beta = 0.0734$  (SE = 0.00005) for  $t \geq 5$  days. The derived relationship does not hold well for  $t < 5$  days because of the delay until the onset of illness in the first cases. In addition, it may not hold for larger values of  $N_0$  and  $t$  where nonlinear mixing patterns and depletion of susceptibles are likely to have an increasingly large effect on  $I(t)$ . A different modeling strategy would probably be required to estimate the potential extent of outbreaks for much larger numbers of initial index cases, but such events are likely to be much less probable.

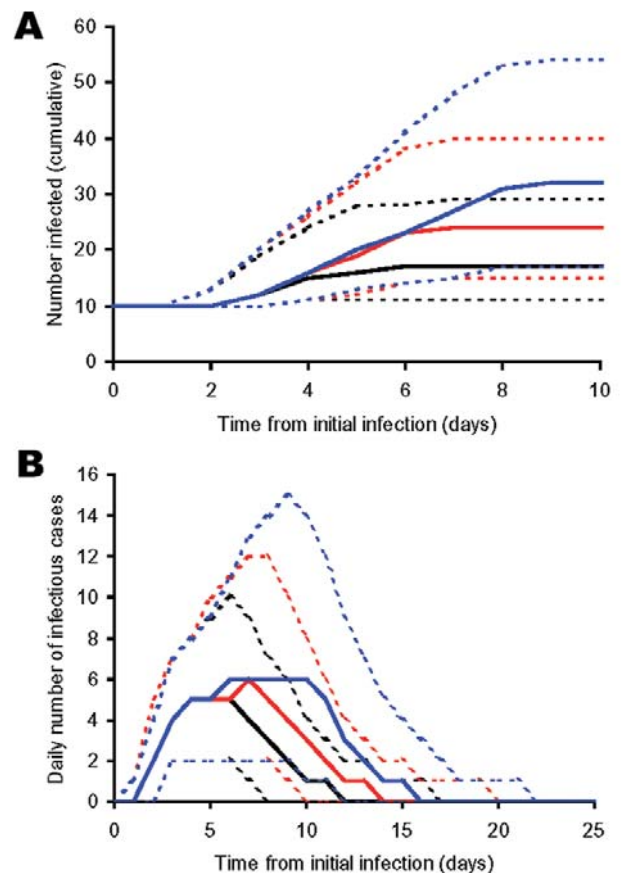


Figure 6. Estimates for (A) the cumulative number of people infected from the time of the first infection, and (B) daily number of infected people, where  $D_0 = 1$  (black), 5 (red) and 10 (blue). Solid lines represent the median number of cases from multiple iterations ( $n = 1000$ ) of the model and the dotted lines give the upper and lower 95th percentiles.

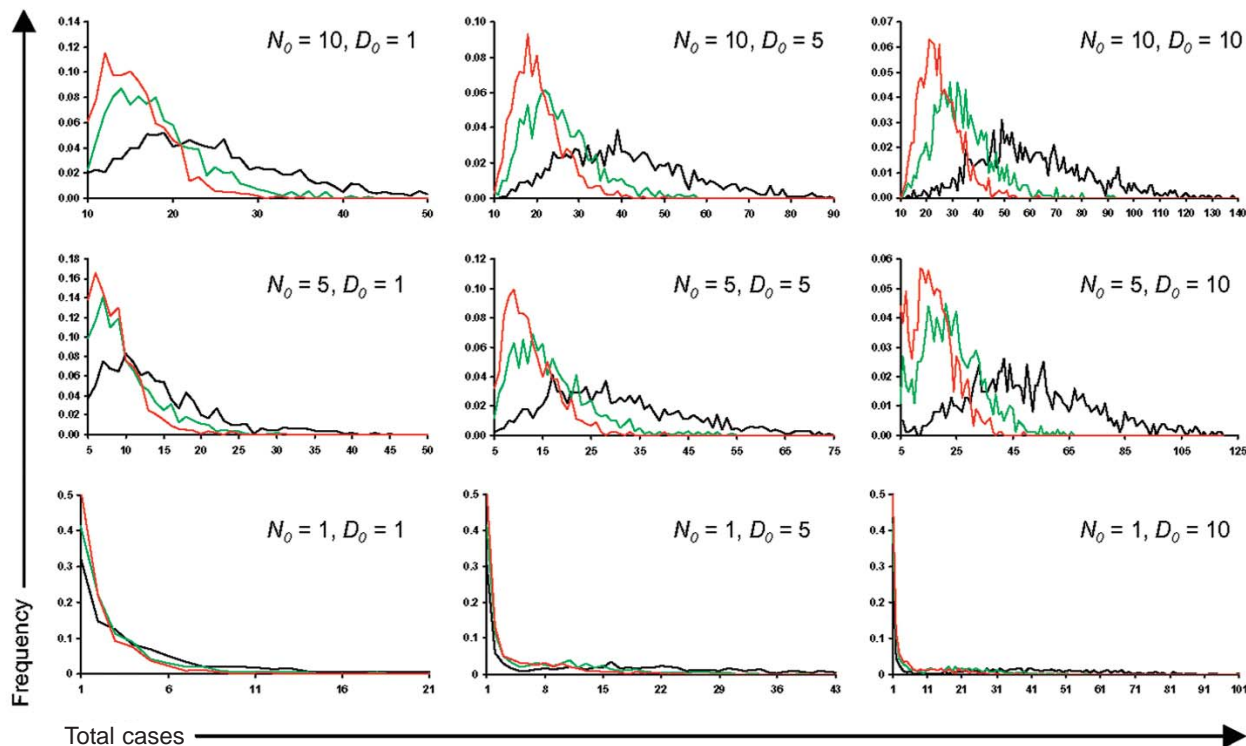


Figure 7. Variation in the expected number of cases at the end of an outbreak when  $N_0$ ,  $D_0$ , and  $R_0$  are varied across multiple iterations ( $n = 27,000$ ) of the model (red denotes  $R_0 = 0.96$ , green denotes  $R_0 = 1.3$ , and black denotes  $R_0 = 2.3$ ). (N.B. Note scale changes).

The transmission rate derived here for primary pneumonic plague is relatively low compared to many other communicable diseases (36), and in 43% of the simulated outbreaks initiated by one index case, no transmission occurred. However, the rapid onset of the infectious period (Figure 1) and the high variance associated with the transmission rate means that if control measures are not promptly and efficiently applied, in some instances much larger outbreaks could occur. For example, for those simulated outbreaks that did “take-off”, large numbers of cases could result before interventions halted further transmission (Figure 5). Small changes in  $D_0$  considerably increased the probability of larger numbers of total expected cases (Figure 5A) and extended the lengths of outbreaks (Figure 5B).

Where  $N_0$  is large (e.g., following an efficient aerosolized release of *Y. pestis*), the dynamics associated with outbreaks will be considerably different than when  $N_0$  is small for 2 key reasons. The first reason is that for large  $N_0$  the probability of transmission is more likely so that natural epidemic die-off will be a less likely event. The second is that outbreak detection will occur more rapidly as it may not be necessary for multiple generations to have occurred before  $D_0$  is reached. Thus, the changes in total numbers of cases per outbreak due to the variation in  $D_0$  are relatively smaller when  $N_0$  is higher (*c.f.* Figures 5 and 6,

and panels in Figure 7) because the difference in the time to  $D_0$  occurring become less as  $N_0$  increases. Thus, for higher  $N_0$ ,  $D_0$  becomes a less significant factor in determining the total number of cases per outbreak. However, for large  $N_0$ , other factors are likely to impact on the control measures, such as limitations in the capacity of healthcare facilities and antimicrobial prophylaxis to cope with large numbers of cases. For large  $N_0$  and larger ensuing outbreaks that might exceed response capacities, the assumption in the modeling here that transmission would be reduced effectively to zero following outbreak detection would have to be reconsidered in the light of resource constraints.

Reducing the average number of secondary cases per primary cases below one is a key step in controlling outbreaks, as this means that the number of new cases declines in successive generations of infection. Since the value of  $R_0$  for primary pneumonic plague is already close to one, the control of potential outbreaks in most cases should be relatively straightforward and undemanding, especially if started by relatively few initial index case-patients. However, given that the upper and lower 95% confidence limits for the estimate of  $R_0$  (based on the significance of the  $\chi^2$ -values derived from minimizing the log-likelihood function) are 2.3 (variance = 7.8) and 0.96 (variance = 1.9), outbreaks with higher values of  $R_0$  in this range could result with greater probability in considerably

large outbreaks that would be increasingly difficult to control unless measures were implemented quickly and efficiently (Figure 7).

The fact that the estimated  $R_0$  is close to one reflects the frequent qualitative observation (23–26,30–31,33) that those infected tend to be those directly caring for ill persons either at home or in a healthcare setting (Figure 4). Given the close contact that was required for transmission and that transmission actually occurred relatively infrequently, the predominating issue determining the variability of transmission between outbreaks is likely to have been stochasticity. This assertion is supported by the results of the simulations, which demonstrate a range of potential sizes and lengths for outbreaks even for individual mean  $R_0$  values (Figures 5 and 7). Although cultural and other factors, such as social and healthcare structures, may well have been different across the outbreaks that have been analyzed, in most cases these factors probably had a relatively minor impact. Although the transmission rate of primary pneumonic plague appears to have been consistently low across these better documented outbreaks, stochastic effects could still generate significant outbreaks by chance (Figures 5 and 7), which coupled with the rapid kinetics of the infection means that such outbreaks could also develop rapidly. In the sensitivity analysis here, however, even such larger outbreaks rarely exceeded more than a hundred cases, even for the higher estimates of  $R_0$ ,  $N_0$ , and  $D_0$ . Of course, this assumes relatively small numbers of initial index cases ( $\sim N_0 \leq 10$ ), relatively sensitive outbreak detection systems ( $\sim D_0 \leq 10$ ), and prompt and efficient public health interventions (transmission tends to zero immediately following outbreak detection). Thus, the key element in the control of smaller outbreaks of primary pneumonic plague would be the acuity of disease surveillance systems and quick detection of outbreaks, the efficiency of which might depend significantly on the number of persons initially infected.

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# Dengue Spatial and Temporal Patterns, French Guiana, 2001

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To study a 2001 dengue fever outbreak in Iracoubo, French Guiana, we recorded the location of all patients' homes and the date when symptoms were first observed. A geographic information system (GIS) was used to integrate the patient-related information. The Knox test, a classic space-time analysis technique, was used to detect spatiotemporal clustering. Analysis of the relative-risk (RR) variations when space and time distances vary, highlighted the maximum space and time extent of a dengue transmission focus. The results show that heterogeneity in the RR variations in space and time corresponds to known entomologic and epidemiologic factors, such as the mosquito feeding cycle and host-seeking behavior. This finding demonstrates the relevance and potential of the use of GIS and spatial statistics for elaborating a dengue fever surveillance strategy.

While investigating the spatial patterning of health events and disease outcomes has a long history (1), the development of geographic information systems (GIS) has recently enabled epidemiologists to include a spatial component in epidemiologic studies more easily. GIS are computer systems that allow the collection, storage, integration, analysis, and display of spatially referenced data. In the field of health, GIS have been widely used for disease mapping of different pathologies, in analysis of space and space-time distributions of disease data (2–5), in identifying risk factors (6–8), and in mapping risk areas (9). In most studies, each patient or person exposed to a disease is located at the residential address, and these locations are integrated into a GIS for mapping and analysis. Because GIS allows epidemiologists to map environmental factors associated with disease vectors, it has become especially relevant for the surveillance of infectious and vector-borne diseases such as malaria (3,8,10) or Lyme disease (11–13).

In particular, GIS and spatial statistics should be useful for surveillance of dengue fever (DF), an arboviral disease

transmitted to humans by mosquitoes of the *Aedes* genus (14). Indeed, because no vaccine or specific treatment is available, the only solution to prevent the disease is vector control strategy. This control strategy requires that risk areas and risk periods be identified. Several studies, some in which GIS was used, have been conducted to identify the mechanisms of the spread of dengue viruses in a community and to improve prevention strategies (4,15–17). The existence of case-clusters inside the same house has often been described (4,15,16,18–24). Moreover, by a space-time analysis of reported dengue cases in Puerto Rico, Morrison et al. have shown the apparent clustering of cases at short distances over brief periods of time (4). Nevertheless, limits of this cluster have not been calculated.

To better understand the transmission dynamics of dengue, we used a GIS to describe the spread of dengue viruses in a small locality. Data were obtained from a recent dengue fever outbreak in Iracoubo, a small town located in French Guiana, an overseas French administrative unit between Suriname and North Brazil.

In French Guiana, DF is recognized as endemic, with dengue epidemics occurring since 1965 at 4- to 6-year intervals (25). The four dengue virus serotypes (DEN-1, DEN-2, DEN-4, and more recently DEN-3) have been isolated. The mosquito *Aedes aegypti* is the only known dengue vector in French Guiana. We report the investigation of space-time patterns of confirmed laboratory-positive and suspected cases; evaluate the efficiency of using GIS technologies in a dengue prevention program, and propose a surveillance strategy.

## Materials and Methods

### Study Site and Population

Iracoubo is a small rural municipality located on the coastal plain of French Guiana with a population of 1,428 inhabitants (26), most of whom live in the main town or in the Bellevue village, located 5 km from the main town (Figure 1). Housing areas are surrounded by rain forest, mangrove forest, and coastal wetlands.

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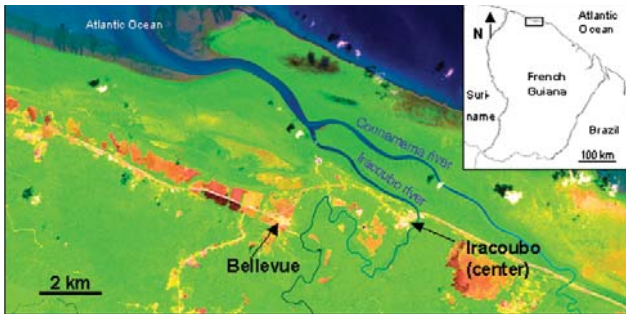


Figure 1. Iracoubo, French Guiana (Landsat TM imagery). Housing areas (pink) are surrounded by coastal wetlands (orange), rain forest and mangrove forests (green areas). Ocean and rivers are in blue.

### Patients

All patients who visited the healthcare center of Iracoubo with a temperature of  $\geq 38.5^{\circ}\text{C}$ , arthralgia, headache, or myalgia, were suspected of having DF. Blood samples were taken for evaluation of probable and confirmed DF cases. The terms suspected, probable, and confirmed cases of DF were used according to the definitions adopted by the Council of State and Territorial Epidemiologists and the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia (27). A suspected case is defined as an illness in a patient whose serum was sent to National Reference Centre for Arboviruses (Institut Pasteur de la Guyane, Cayenne, French Guiana) for the diagnosis of DF. A probable case was an illness in a person that is clinically compatible with dengue, combined with supportive serologic test results (a single convalescent-phase serum specimen containing dengue virus immunoglobulin [Ig] M antibody, or a dengue virus IgG antibody titer of  $\geq 1,280$  by hemagglutination inhibition assay [HI]). A confirmed case was defined as having any of the following criteria: isolation of dengue virus from serum, demonstration of a dengue virus cDNA fragment by amplification (reverse transcription–polymerase chain reaction [RT-PCR]) from a serum sample, IgM antibody seroconversion, or a fourfold or greater increase in reciprocal titers of IgG antibody to one or more dengue virus antigens in paired serum samples.

During a dengue epidemic in a disease-endemic area such as French Guiana, the predictive positive value for a probable dengue case to be a confirmed case is very high (24). For this reason, we decided to include the probable dengue cases in the group of confirmed cases. Thus, we use the term confirmed case for both probable and confirmed dengue cases, and the term suspected case for all reported cases during the epidemic.

### Laboratory Diagnosis

All tests were performed at the Institut Pasteur de la

Guyane, National Reference Centre for Arboviruses (Cayenne, French Guiana).

### Serologic Tests

Two techniques were used to detect antibodies to dengue viruses. The first was detection of IgM dengue virus antibodies by using an IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) with a tetravalent dengue virus antigen. The procedure was modified from a previously described method (28).

HI was also used. HI titers were determined by using the method of Clarke and Casals (29) that was adapted to a microtechnique. Antibody responses to dengue virus were interpreted according to the World Health Organization criteria (30).

### Virus Isolation and Identification

Acute-phase serum samples from febrile patients ( $< 4$  days after the onset of fever) were diluted 10-fold in Leibowitz medium containing 3% fetal calf serum, and dilutions were injected into subconfluent AP 61 cell cultures as previously described (31). After 7 days of culture, cells were harvested, and dengue viruses were identified according to serotype by an indirect immunofluorescence assay (IFA) with monoclonal antibodies specific to DEN-1, -2, -3, and -4 viruses (provided by CDC, Fort Collins, CO).

### Detection of Dengue Viruses by Using RT-PCR Analysis

Viral RNA was extracted from a 50- $\mu\text{L}$  aliquot of acute-phase serum with TRIzol (Invitrogen Life Technologies, Paisley, Refrewshire, UK), according to the manufacturer's recommendations and precipitated with isopropanol and 1  $\mu\text{L}$  of glycogen (5  $\mu\text{g}/\mu\text{L}$ ) (Roche Diagnostics, Mannheim, Germany). Air-dried RNA pellets were suspended in 20  $\mu\text{L}$  of water. Then, 5  $\mu\text{L}$  of RNA were mixed with 200 ng of random hexamer primers, and first-strand cDNA synthesis was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), according to the manufacturer's recommendations. The first run of RT-PCR analysis and subsequent seminested PCR analysis were performed following a previously described procedure (32).

### Cases Georeferencing

For all suspected dengue patients, patient's home was recorded with a cadastral map (paper copy, scale 1/1,000). Georeferenced aerial photographs were used to improve the identification and the location. The geographic coordinates were integrated into a GIS (Geoconcept software) (33), with the following information about the patient: identification number, date of onset of symptoms, age, sex, diagnosis.

### Spatial and Temporal Patterns Analysis

Assuming that DF spread within a community leads to the creation of transmission focus, the distance between neighboring housing would be an important factor in the spatial extension of these foci. We used the GIS and geocoded aerial photographs to locate all houses and to calculate the mean distance between neighboring houses (Geoconcept software) (33).

We used the Knox (34) test to identify possible space-time interactions, i.e., to determine whether cases which are close in distance will also be close in time. This method evaluates whether the number of pairs of cases found at a fixed temporal and spatial distances is substantially different from the number of pairs of cases expected at these distances by chance, when the times of occurrence of cases are randomly distributed across the case locations.

The ratio between real number of pairs of cases found at the space-distance  $s$  (in meters) and the time-distance  $t$  (in days) and the number of pairs of cases found at these distances by chance could be considered as the RR of occurrence of another dengue case,  $t$  days later and  $s$  meters away from the first case of dengue.

The Knox test was first computed for the population of patients with confirmed cases and for the population of those with suspected cases. Results were calculated for time distances varying from 1 to 200 days (duration of the epidemic) by 1-day step and spatial distances varying from 5 to 6,500 m (step: 5 m). An "RR map" was then obtained by interpolating the significant values ( $p = 0.05$ ) (Surfer software) (35).

The final result is a representation of the RR, when space-distance and time-distance from a hypothetical dengue patient vary. The correlation between RR values derived from the confirmed cases and those derived from the suspected cases was evaluated.

## Results

### Serologic Tests

In Iracoubo center and Bellevue, 161 patients with suspected dengue cases were reported between April and November 2001, which corresponds to 11.3% of the population. Blood samples from 57 patients were analyzed in the National Reference Center for Arboviruses, Arbovirology Laboratory of the Pasteur Institute of French Guiana. Among the 57 patients, 32 cases of DF were confirmed (56.1%). A total of 25 suspected cases were not confirmed; among them 4 cases were indeterminate and 21 were negative (Table). Virus isolation results show that the majority of confirmed cases were caused by DEN-3 (90%).

Table. Serologic analysis results

Cases	Iracoubo center	Bellevue	Total
Reported	93	69	162
Analyzed	34	23	57
Laboratory-negative	14	7	21
Indeterminate	1	3	4
Laboratory-positive	19	13	32
Confirmed	14	8	22 <sup>a</sup>
Probable	5	5	10

<sup>a</sup>19 DEN-3; 2 DEN-1, 1 seroconversion.

### Epidemic Description

The first suspected dengue case was reported on April 10 but samples were not analyzed. The first confirmed dengue case occurred on April 22. Then, the epidemic spread rapidly through the community (Figure 2), with a temporal lag between cases occurring in Iracoubo center and those occurring in Bellevue (Figure 3). Indeed, 100% of the confirmed cases in Iracoubo center occurred between April and July, whereas 76.9% of confirmed cases in Bellevue occurred in October and November. The first glimpse of the spatial distribution of confirmed and suspected cases shows the existence of apparent spatial clusters (more than 2 confirmed cases or 3 suspected cases in the same neighborhood) (Figure 2).

### Spatial-Temporal Patterns Analysis

We considered 406 buildings in the calculation. The mean distance between adjacent houses was 24.6 m for the whole municipality, including means of 22.6 m for Iracoubo center and 29.6 m for Bellevue.

A first analysis of the RR variations when space and time distances vary over all the epidemic's extent high-



Figure 2. Maps showing locations of dengue patients in Iracoubo center (weeks 3, 6, 9, 12), showing locations of confirmed (red) and suspected (blue) dengue fever patients. Black circles correspond to spatial clusters, that is, neighborhoods where more than two confirmed cases or three suspected cases occurred.

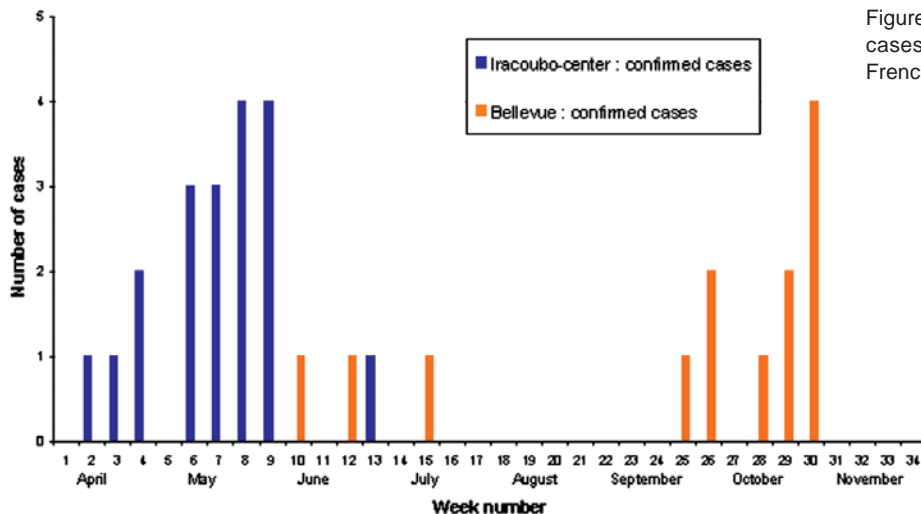


Figure 3. Number of confirmed dengue fever cases per week, Iracoubo municipality, French Guiana, April–November 2001.

lights a main risk area, with  $RR > 1$  ( $p < 0.001$ ) (Figure 4). This area corresponds to a substantial increase in the theoretical risk of the occurrence of another dengue case. This area is active inside the boundaries of 400 m and 40 days.

A more detailed analysis of this risk area shows a strong heterogeneity: an area is very high risk ( $RR > 5$ ) at short distances (15 m) and over brief periods (6 days). Beyond these space-time limits, the RR rapidly decreases (Figure 5). Moreover, particular patterns are observed, like a temporal periodicity, with peaks of risk every 3 days (Figure 6A). Spatial breaks seem to appear at the approximate distances 20–25 m, 45–50 m, and 80–85 m (Figures 5 and 6A), showing three different risk levels (Figure 5).

A strong concordance exists between the results obtained by using the dengue laboratory-positive cases and those obtained by using all suspected cases: the space and time boundaries are roughly the same (Figure 6B). Although the RR values are different for the same space and time distances, they are correlated with a high correlation coefficient ( $r = 0.93$ ;  $p < 0.05$ ).

**Discussion**

To study the dynamics of a DF outbreak in the small municipality of Iracoubo during 2001, we located all patients in space by determining their home address and in time by obtaining the date of onset of symptoms. Although the definition of time-location is obvious, the definition of space-location can be questioned. Indeed, using this factor implies that patients have contracted the disease at home, which is a strong hypothesis. This hypothesis is based on practical constraints (since the residential address is the easiest way to implement a location criterion), and on the results of several studies confirming that dengue risk exposure is more important at home because female *Aedes aegypti* mosquitoes are endophilic and take their blood meal during the day with often a peak in the early morning

and in the evening (36), and even sometimes during the night (37,38).

The difficulty of locating each patient’s home has to be pointed out, however. Previous studies had to face the major problem of locating each address and verifying it in the field, which requires a substantial time investment (4). For our study in Iracoubo, the relatively small group of patients was easily and quickly located by using maps and

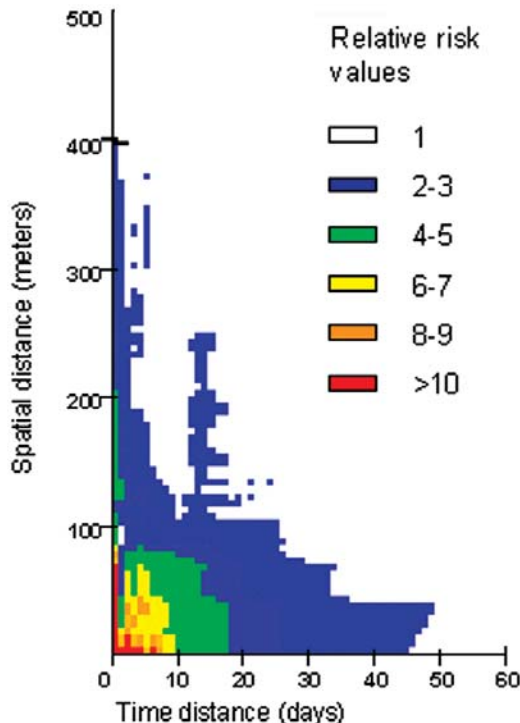


Figure 4. Global representation of the relative-risk (RR) calculated from the confirmed cases data, when space-distance and time-distance from a first theoretical dengue case vary respectively from 0 to 500 m and from 0 to 60 days. Color indicates RR values greater than one ( $p < 0.001$ ). High RR values are in red.



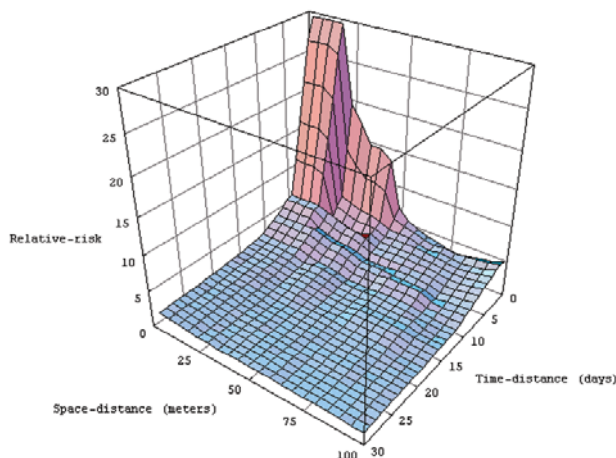


Figure 5. Three-dimensional representation of the main risk area for dengue fever (within 100 m and 30 days' boundaries) derived from data on confirmed cases.

aerial photographs. Nevertheless, in the context of an operational dengue surveillance system deployment, our alternative to address georeferencing is not adapted. Therefore, an original interactive software for georeferencing cases by using aerial photographs and maps, during the consultation by the physician or in healthcare centers, was implemented in French Guiana (DOC\_teur Software) (39). This could be an alternative solution for the problem of georeferencing cases, provided that healthcare centers have computer capabilities.

An initial interpretation of the spatial dengue distribution shows that all areas of the municipality were rapidly affected by the disease. Moreover, the distribution highlights spatial case-clusters inside individual houses and in the nearby neighborhoods of case-patients (Figure 2). One of the aims of the spatial and temporal patterns analysis was to clarify this qualitative interpretation.

Our study on space-time patterning led us to map in space and time the RR for DF within a particular space-time window from the first hypothetical suspected case. This RR index map allowed us to determine the boundaries in space and time of the maximum dengue transmission focus extent (400 m, 40 days) and to identify a very high-risk area at a short distance (15 m) over a short period (6 days). These results confirm the focal nature of DF as reported in the literature, and, above all, fix quantitative values for the transmission focus limits.

Moreover, the strong heterogeneity apparent in the RR index map (Figure 6) is coherent with known entomologic and epidemiologic factors. Indeed, the marked 3 days periodicity is consistent with the length of the gonotrophic cycle of the female *Ae. aegypti* mosquito (36). After being fed and achieving extrinsic incubation, a mosquito bite would be infectious and lead to a human dengue case after

the intrinsic incubation period; whether the mosquito bites every 3 days and whether we assumed that intrinsic incubation period is constant in duration, then other dengue cases would appear every 3 days.

On the other hand, spatial breaks in the disease occurrence seem to correspond roughly to the spatial distances between houses as determined with aerial photographs. Indeed, aerial photo-interpretation shows that for each house, the direct neighboring house is included, in average, in a 25-m radius, which also includes the risk area shown by our results for dengue occurrence. The two next distance peaks, namely 45 m and 80 m, correspond to the third and fourth nearest areas of housing, respectively.

Those similarities between patterns in the RR map derived from space-time location of dengue cases and known transmission factors confirm the relevance of using GIS for the epidemic description. In particular, the available data seem consistent with the hypothesis that most people were infected at home or near the home during the Iracoubo epidemic.

In future studies, obtaining the exact incidence in the exposed population will be preferable. For this goal, a prospective seroepidemiologic study must be conducted in the overall exposed population to identify all dengue cases, including the asymptomatic cases. This kind of study would certainly increase the accuracy of the GIS for the epidemic description. In Iracoubo the distribution of the nonsymptomatic cases and the nonreported cases likely paralleled the spatial distribution of the reported cases. Thus, the fact that we did not dispose of the total number of dengue cases induced more likely a decrease in the precision, than an inaccurate representation of dengue transmission. This hypothesis will be tested in a future study.

These first results show that an objective description of a dengue virus spread using GIS and space-time statistics allows epidemiologists to define risk areas and risk periods, which are necessary for implementing an efficient

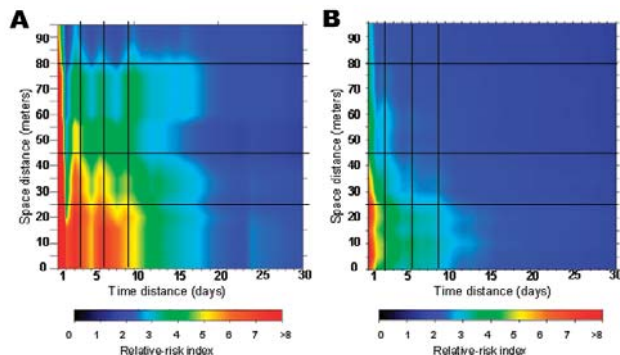


Figure 6. Main risk area for dengue fever (within 100 m and 30 days' boundaries), derived from laboratory-positive cases data (A) and all suspected cases data (B). Vertical dark lines indicate an apparent temporal periodicity, and horizontal dark lines correspond to apparent spatial breaks.

surveillance strategy. Moreover, the strong concordance of the two RR maps derived from the confirmed cases and suspected cases indicates that a surveillance program could be based on information concerning all suspected cases. Including such information would allow a better response to an outbreak.

Analyzing RR representation shows a very high risk area 6 days after and at <15 m from a first hypothetical dengue case (Figure 5). Because of the short duration of the dengue intrinsic incubation period, each dengue patient contracted the disease a few days before its clinical expression. As a consequence, there were no means of reducing the first RR peak after the first dengue case was detected. Nevertheless, vector control could have reduced the secondary RR peaks, which occurred 3, 6, and 9 days after the first high RR area was identified (Figure 6).

These results could increase the efficiency of the vector-control strategy. Indeed, the RR representation indicates that vector control should be more efficient when conducted inside the houses and against adult mosquitoes. All houses inside a 100-m neighborhood should be treated. The distance of 100 m corresponds to a statistical threshold, which is a result of our study: outside of 100 m around the dengue focus, the probability of observing a dengue case is low. However, if the number of cases reported increases, we will likely increase the precision of such reporting, and this threshold could vary. If these results are confirmed in the future, this would likely lead to improvements in indoor vector control (by indoor spraying of insecticide) during dengue epidemics, in particular in the houses near a house where a confirmed or suspected case occurred, provided that the mosquitoes have been shown to be sensitive to the insecticide. Reducing breeding sites and increasing indoor vector control could be the major means of controlling dengue spread during an epidemic.

## Conclusion

The use of a GIS in a dengue surveillance program requires an efficient case location system and a concerted effort by all health stakeholders: physicians, hospitals, pathology laboratories, and vector control agencies. In French Guiana, a research program named S2Dengue (Spatial Surveillance of Dengue) joins the different health stakeholders for the real-time collection of all dengue-related information (suspected and confirmed cases, vector densities, etc.). The first objective of this project is to provide all participants with weekly maps of dengue incidence to improve prevention measures. The second objective is to link this information with relevant environmental factors and establish a model of the epidemic dynamics.

This program will allow us to validate our results concerning the characteristics of the dynamic of dengue in

French Guiana and confirm the potential of using geographic information systems for dengue surveillance at a country level. This effort will also contribute to dengue control strategy.

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# *Babesia divergens*-like Infection, Washington State

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Most reported U.S. zoonotic cases of babesiosis have occurred in the Northeast and been caused by *Babesia microti*. In Washington State, three cases of babesiosis have been reported previously, which were caused by WA1 (for “Washington 1”)–type parasites. We investigated a case of babesiosis in Washington in an 82-year-old man whose spleen had been removed and whose parasitemia level was 41.4%. The complete 18S ribosomal RNA gene of the parasite was amplified from specimens of his whole blood by polymerase chain reaction. Phylogenetic analysis showed the parasite is most closely related, but not identical, to *B. divergens* (similarity score, 99.5%), a bovine parasite in Europe. By indirect fluorescent-antibody testing, his serum reacted to *B. divergens* but not to *B. microti* or WA1 antigens. This case demonstrates that babesiosis can be caused by novel parasites detectable by manual examination of blood smears but not by serologic or molecular testing for *B. microti* or WA1-type parasites.

Hundreds of zoonotic cases of babesiosis have been reported in the United States, approximately 30 in Europe, and a few elsewhere (1–14). Most of the reported U.S. cases have been caused by *Babesia microti*, a parasite of small mammals transmitted by *Ixodes scapularis* ticks, and have occurred in the Northeast or, less commonly, the upper Midwest (4).

Few zoonotic cases of babesiosis have been reported in the western United States (7–11). Specifically, in Washington State, only three cases, two presumably tick-borne and one associated with blood transfusion, have been reported previously (7,8,10). The index tick-borne case occurred in 1991 in Klickitat County, in south-central Washington (7,8). The other two cases occurred in 1994: one in a person transfused with infected erythrocytes and the other in the implicated blood donor, who lived in King County, in the western foothills of the Cascade Mountains (10). All three of these cases were caused by WA1 (for

“Washington 1”)–type parasites (1,2,7–10,14) (Figure 1). However, the etiologic agent of the case of babesiosis in Washington in 2002 that we describe here is most closely related, by molecular criteria, to *B. divergens*, a bovine parasite in Europe (5,6).

## Methods

### Serologic Testing

Serum specimens were tested at the Centers for Disease Control and Prevention (CDC), in serial fourfold dilutions, by indirect fluorescent-antibody (IFA) testing,

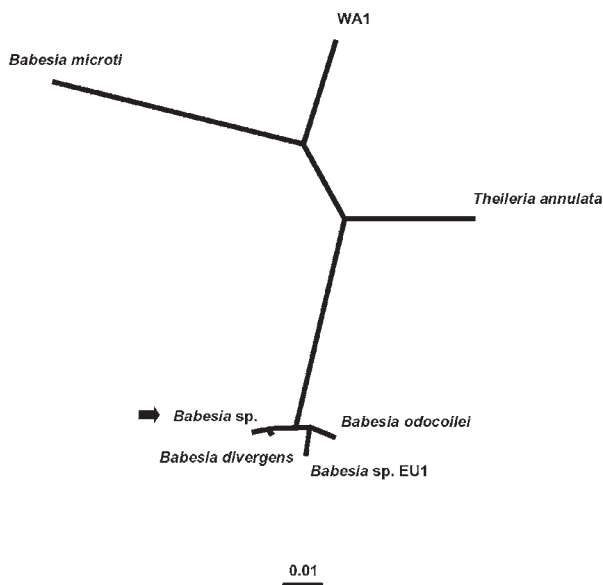


Figure 1. Unrooted phylogenetic tree for the complete 18S rRNA gene of selected *Babesia* spp. The tree was computed by using the quartet puzzling maximum likelihood method of the TREE-PUZZLE program. The scale bar indicates an evolutionary distance of 0.01 nucleotide substitutions per position in the sequence. The GenBank accession numbers for the sequences used in the analysis are as follows: *B. divergens* (6), AY046576; *B. odocoilei*, AY046577; *Babesia* sp. EU1 (6), AY046575; the *Babesia* sp. from the patient in Washington State, AY274114 (see arrow); *B. microti*, U09833; WA1, from the index case of infection with WA1-type parasites (7,8), AF158700; and *Theileria annulata*, M64243.

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for reactivity to *B. microti* (15), WA1 (7), and *B. divergens* antigens (6). The antigen sources were human isolates of *B. microti* and WA1 and a bovine isolate of *B. divergens* (Purnell strain [16]), which had been passaged in gerbils (Mongolian jirds; *Meriones unguiculatus*) and adapted to culture in bovine erythrocytes (17).

**Animal Inoculation**

To attempt to obtain an isolate of the parasite that infected the patient (hereafter referred to as the patient's parasite), whole blood specimens, collected from him in tubes containing the anticoagulant disodium EDTA, were

injected into hamsters (*Mesocricetus auratus*) and jirds. Animal experimentation guidelines were followed. Both hamsters and jirds are competent hosts for *B. microti* and WA1-type parasites, and jirds are competent hosts for *B. divergens* (18). No pretreatment specimens were available. Refrigerated specimens collected on July 31, 2002 (the date antibabesial therapy was begun [Table 1]), and August 1 were each injected intraperitoneally into two hamsters (0.25 mL and 0.5 mL/animal, respectively) on August 2. A specimen obtained on September 10 (15 days after therapy was discontinued) was injected intraperitoneally into two jirds (1 mL/animal) the next day. The animals were

Table 1. Clinical data on selected dates for a patient in Washington State infected with a *Babesia divergens*-like parasite, 2002<sup>a</sup>

Date	Temperature (°C)	Hematocrit (%) <sup>b</sup>	Leukocyte count (10 <sup>9</sup> /L)	Platelet count (10 <sup>9</sup> /L)	Parasitemia level (%) <sup>c</sup>	Creatinine level (mg/dL) <sup>d</sup>	Total/direct bilirubin levels (mg/dL)	Lactate dehydrogenase level (U/L)	Comments
July 30	38.5	43	19.6	34	25.5 <sup>e</sup>	8.5	10.2	4,283	Admitted to a community hospital
July 31 <sup>e</sup>		40	21.2 <sup>f</sup>	21	41.4 <sup>e</sup>	10.3	8.2/2.9		Babesiosis diagnosed; antibabesial therapy started (see text) <sup>g</sup>
July 31 <sup>h</sup>	39.9	36	18.5	25	28.2	11.1 <sup>i</sup>	9.9/3.1	6,674	6 U platelets transfused
August 1	37.7	27	21.6	57	24.7	6.4	6.7/2.3	2,898	Began hemodialysis; 2 U packed erythrocytes transfused
August 2	36.9	32	22.1	67	17.9	4.2	3.3/2.1	5,802	
August 3	37.5	29	17.2	96	13.6	7.3		2,423	
August 4	36.9	27	10.4	110		6.4	1.3/0.7		
August 5	37.6	26	9.9	135	11.9	9.0			
August 6	37.5	24	9.5	149	13.2	11.0			2 U packed erythrocytes transfused
August 7	37.5	30	9.9	149		8.8			
August 8	37.9	30	9.2	204	7.7	11.2			
August 9	36.7	28	9.2	161 <sup>j</sup>		8.2			Discharged home
September 13		28	7.3	179		2.8			
November 1		39	6.0			1.7			

<sup>a</sup>Normal ranges for laboratory values at community hospital (July 30–31): creatinine, 0.7–1.5 mg/dL; total bilirubin, 0.1–1.0 mg/dL; lactate dehydrogenase, 100–200 U/L. Normal ranges at University of Washington Medical Center (July 31–Aug. 9): creatinine, 0.3–1.2 mg/dL; total bilirubin, 0.1–1.0 mg/dL; direct bilirubin, 0.0–0.3 mg/dL; lactate dehydrogenase, 0–190 U/L.

<sup>b</sup>Hematocrit normalized to values of 42% (Feb. 3, 2003) and 45% (June 27, 2003).

<sup>c</sup>All parasitemia levels were determined by the same person at the Centers for Disease Control and Prevention. Level for July 30 was determined from a peripheral smear made the next day (i.e., blood not fresh). The other pretreatment parasitemia level (i.e., from July 31 at community hospital) was determined from a smear made from fresh blood.

<sup>d</sup>Baseline and posthospitalization creatinine values include: 1.6 mg/dL (Sept. 2001), 1.7 mg/dL (Feb. 3, 2003), and 1.6 mg/dL (June 27, 2003).

<sup>e</sup>Data obtained at community hospital.

<sup>f</sup>Leukocyte differential: 74% segmented neutrophils, 9% lymphocytes, 8% atypical lymphocytes (including immunoblasts and plasmacytoid forms), 7% monocytes, 1% eosinophils, and 1% metamyelocytes.

<sup>g</sup>On Aug. 1, dosage regimen changed from clindamycin (1.2 g twice daily, by IV infusion) and quinine sulfate (650 mg thrice daily, by mouth), to clindamycin (600 mg four times daily, by IV infusion) and quinine (650 mg daily, by mouth). After patient was discharged from hospital on Aug. 9, therapy continued through Aug. 26; regimen: clindamycin (600 mg four times daily, by mouth) and quinine (650 mg daily, by mouth). Patient also treated with erythropoietin until Nov. 2002.

<sup>h</sup>Data obtained at University of Washington Medical Center.

<sup>i</sup>Dipstick analysis of urine specimen showed 3+ protein, 3+ blood, 3+ bilirubin, and 1+ ketones; and was positive for nitrites and leukocyte esterase. Microscopic examination showed granular casts and 1+ leukocytes. Culture demonstrated >10<sup>5</sup> colonies of *Escherichia coli* per milliliter of urine. The urinary tract infection was treated with ceftriaxone.

<sup>j</sup>Value could be inaccurate; platelets clumped on blood smear.

monitored for parasitemia, weekly for 8 weeks, by examination of Giemsa-stained smears of blood obtained by tail snip (hamsters) or toenail clip (jirds). Blood obtained from the animals after the 8-week monitoring period was examined by polymerase chain reaction (PCR; see next section).

#### DNA Extraction, Amplification, and Sequencing

DNA was extracted at CDC from whole blood specimens collected from the patient in EDTA tubes, by using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA); the DNA was stored at 4°C. The complete 18S ribosomal RNA (18S rRNA) gene from the patient's parasite was amplified by PCR, with a pair of apicomplexan 18S rRNA-specific primers: CRYPTO5 (5'-AACCTGGTTGATCCTGCCAGT-3') and CRYPTO3 (5'-GCTTGATCCTTCTGCAGGTTACCTAC-3'). PCR was conducted with AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). The conditions for PCR included 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 1.5 min. Final extension was done at 72°C for 9 min, followed by a hold step at 4°C. The amplification product was purified by using the StrataPrep DNA Purification Kit (Stratagene, La Jolla, CA).

Both strands of the PCR product were sequenced by using a set of internal primers. Sequencing reactions were performed with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), and reactions were analyzed on the ABI 3100 automatic DNA sequencer (Applied Biosystems). The resulting sequences were assembled by using the program SeqMan II (DNASTAR, Inc., Madison, WI). The GenBank accession number for the sequence obtained by these methods for the patient's parasite is AY274114.

#### Phylogenetic Analysis

The complete sequences of the 18S rRNA genes for *B. divergens*; *B. odocoilei*, a parasite of white-tailed deer (*Odocoileus virginianus*) (17,19); *Babesia* sp. EU1 (for "European Union 1") (6); *B. microti*; the WA1 parasite from the index case of such infection (7,8); and *Theileria annulata* were retrieved from the GenBank database (Figure 1). The sequences were aligned with the sequence for the patient's parasite by using the program Clustal W, version 1.83 (20). Phylogenetic analysis was performed with the following programs: the PHYLIP package, which includes versions 3.573c of CONSENSE, DNADIST, DNAML, NEIGHBOR, and SEQBOOT (21); and version 5.1 of TREE-PUZZLE (22). The phylogenetic trees inferred by these programs were drawn with the program TreeView, version 1.6.6 (23).

#### Case Report

On July 30, 2002, an 82-year-old man in Kitsap County (a peninsula of estuarine lowlands in Puget Sound, in western Washington), was admitted to a community hospital. During the previous 4 days, he had noted the gradual onset of chills (without fever), weakness, malaise, anorexia, dysphagia, marked thirst, and urinary dribbling. Until April or May 2002, he had been an avid jogger. Although he had not had the energy to jog thereafter, he had continued to walk his dog daily; they walked on a road around a lake that abutted his backyard, which he kept "natural," and on a neighbor's wooded property. He had an outdoor cat; had occasionally noted deer and (in previous years) voles or shrews in his yard; did not live near cattle; and had not traveled outside of Kitsap County or Mason County, the adjacent southern county, in the previous couple of years. He had not noted ticks on his body or received blood transfusions. His medical history included longstanding hypertension; secondary renal insufficiency; and incidental splenectomy in approximately 1990, when his distal pancreas, which had a benign mass, was removed.

When he was hospitalized on July 30, his temperature was 38.5°C; pulse, 76 beats/min; blood pressure, 168/94 mm Hg; and respiratory rate, 18 breaths/min. He had scleral icterus and dry mucous membranes from severe dehydration. The salient laboratory data and information about his clinical course are provided in Table 1. Initial laboratory testing showed many abnormalities, including marked thrombocytopenia and elevated values of creatinine, bilirubin, and lactate dehydrogenase.

Babesiosis was diagnosed, when a peripheral smear made on July 31 from blood collected on July 30 was noted to have intraerythrocytic, ring-like trophozoites; the level of parasitemia on a smear of fresh blood from July 31 was 41.4% (Table 1; Figure 2). On July 31, antibabesial therapy was begun at the community hospital; he received one dose of clindamycin (1,200 mg, by intravenous infusion) and one dose of quinine sulfate (975 mg, by mouth; the intended—i.e., ordered—dose was 650 mg).

Later on July 31, he was transferred to the University of Washington Medical Center. He continued therapy with clindamycin and quinine, with periodic adjustments of the dosage regimen (Table 1 footnote). In addition, he was transfused with 6 U of platelets and 4 U of packed erythrocytes. His acute renal failure (Table 1) was treated with hemodialysis; it was attributed to acute tubular necrosis from dehydration and intravascular hemolysis, superimposed on chronic renal insufficiency from hypertensive nephrosclerosis. His hospital course was also notable for laboratory evidence, on August 1, of asymptomatic, subendocardial ischemia and for pulmonary edema.

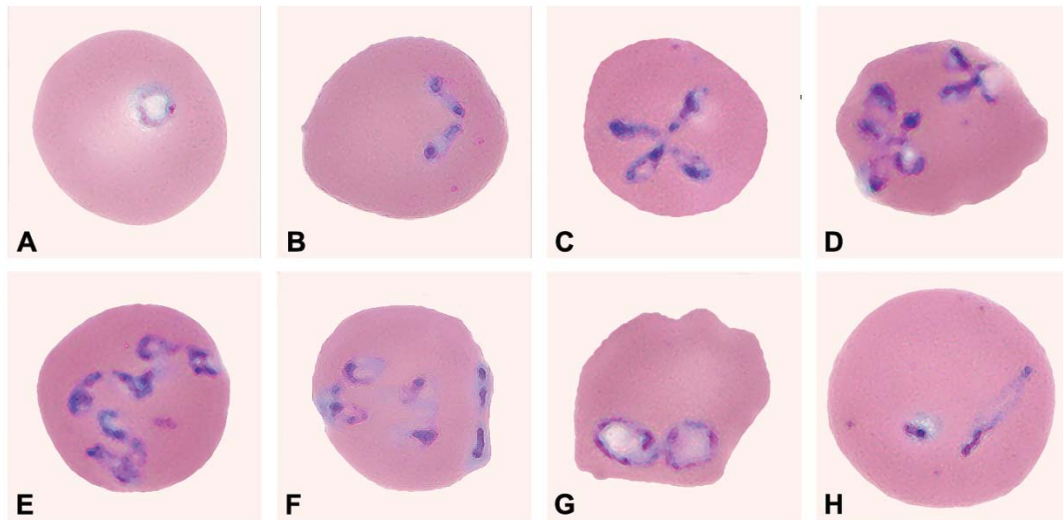


Figure 2. Panel of *Babesia*-infected erythrocytes photographed from pretreatment, Wright's-Giemsa–stained smears of fresh blood obtained from the patient on July 31, 2002. The mean corpuscular volume of the erythrocytes was 103 (normal range 80–100  $\mu\text{m}^3$ ). Note the multiply infected erythrocytes; the pleomorphism of the parasite; and the obtuse (divergent) angle formed by some of the paired structures, which, like the form in (F), is characteristic of *B. divergens* and related parasites isolated from various wild ruminants. The forms of the parasite shown in the panel include: (A) ring-like trophozoite; (B) paired merozoites; (C) maltese-cross (tetrad); (D) various dividing forms; (E) multiple merozoites; (F) appliqué (accolé) form on right border of the erythrocyte; (G) and (H) degenerate (crisis) forms. A glass slide of a peripheral blood smear from July 31 has been deposited in the U.S. National Parasite Collection, Beltsville, Maryland; the accession number (USNPC #) for the slide is 093041.00.

After he was discharged from the hospital on August 9, he continued antibabesial therapy through August 26 (Table 1) and hemodialysis through early September. He resumed jogging and walking in early October 2002 and had remained well as of July 2003.

## Results

### Serologic Testing and Animal Inoculation

The patient's serum did not react to *B. microti* or WA1 antigens but showed marked IFA reactivity to *B. divergens* antigens, which slowly decreased during the 9-month monitoring period (Table 2). Attempts to obtain an isolate of the patient's parasite, by injecting specimens of his blood into hamsters and jirds, were unsuccessful (Table 2 footnote). PCR analysis of blood from the inoculated animals, after they had been monitored for 8 weeks, was negative.

### Molecular Findings

Amplification of the complete 18S rRNA gene of the patient's parasite yielded a specific product of approximately 1,700 bp. Sequence analysis showed that the gene was 1,728 bases long. The sequences of the PCR products from three blood specimens from the patient (Table 2), each processed separately, were identical. The DNA sequence also was verified by staff in an independent laboratory, who had never worked with *B. divergens* or DNA extracted from it.

BLAST search confirmed that the sequence for the patient's parasite was not identical to any complete 18S rRNA sequence in GenBank. The highest similarity score (99.5%) was with *B. divergens* (GenBank no. AY046576); the sequences for *B. divergens* and the patient's parasite differ by eight bases.

In phylogenetic analysis (Figure 1), the patient's parasite clusters together with *B. divergens*. This group forms a sister group to a cluster that includes *B. odocoilei* and *Babesia* sp. EU1 (6). The clustering of the organisms was the same, regardless of the taxonomic method used. The alignment of the sequences used to construct the phylogenetic tree (Figure 1), after columns with gaps and unresolved characters were removed, had 1,651 columns; the bases in 255 columns differed among the *Babesia* spp. included in the analysis. The alignment may be requested from the authors. Serial PCR and IFA data (Table 2) showed that the patient had subpatent parasitemia and a persistently high IFA titer (1:1,024) for at least 2 months after his antibabesial therapy was stopped.

## Discussion

This case of babesiosis had several unusual features. First, it occurred in Washington State, rather than in the Northeast, where most of the reported U.S. cases of zoonotic babesiosis have occurred. Our case raises the count for reported cases of babesiosis in Washington from three to four (one bloodborne and three presumably tick-

Table 2. Results of testing of serial specimens obtained from a patient in Washington State infected with a *Babesia divergens*-like parasite<sup>a</sup>

Date <sup>b</sup>	Examination of smears of whole blood for <i>Babesia</i> parasites	IFA titers to <i>B. divergens</i> antigens <sup>c</sup>	PCR
July 31, 2002	Positive (parasitemia level, 28.2%)	1:64	Positive
September 10	NPF	1:1,024	Positive
October 25	NPF	1:1,024	Positive
November 8	NPF	1:256	Negative
December 12	NPF	1:256	Negative
April 14, 2003	NPF	1:256	Negative

<sup>a</sup>IFA, indirect fluorescent antibody; PCR, polymerase chain reaction; NPF, no parasites found.

<sup>b</sup>See Table 1 for values of other laboratory testing performed on or near dates listed here. Patient received antibabesial therapy from July 31 (before July 31 specimen obtained) through August 26, 2002. Hamsters injected with patient's blood obtained July 31 and August 1 did not become demonstrably parasitemic, nor did jirds injected with blood from September 10.

<sup>c</sup>All of the serum specimens were tested at the Centers for Disease Control and Prevention on same day.

borne cases). Second, the case was caused by a parasite most closely related, by molecular criteria, to *B. divergens*, a European bovine parasite (24), rather than to WA1-type parasites, which caused the three previously reported cases of babesiosis in the state. Third, the patient whose case we describe survived, despite having multiple risk factors for severe babesiosis and death: he was elderly (82 years old), was asplenic, had a high level of parasitemia (41.4%), and had multiorgan dysfunction that included renal failure.

Few cases of babesiosis in the western United States have been reported previously; all occurred in Washington or California. They include two tick-borne cases in California in 1966 (25) and 1979 (26), as well as seven tick-borne and two blood-transfusion-associated cases in California and Washington from 1991 to 2000 (7–11). Whereas the etiologic agents of the cases in 1966 and 1979 were not determined, the last nine cases were caused by the CA1- and WA1-types of *Babesia*- (or *Babesia*-like) piroplasms, which are distinct from each other but in the same phylogenetic group. Although the appropriate position for this clade in phylogenetic analyses of the piroplasms remains unclear (14), the position is remote from *B. microti* and *B. divergens* (11,14) (Figure 1).

The molecular characterization of our patient's parasite (Washington, 2002) showed that the sequence for the complete 18S rRNA gene differs by eight bases from that of *B. divergens* (similarity score, 99.5%). In addition, serologic (Table 2) and morphologic (Figure 2) data support the conclusion that the patient was infected with a *B. divergens*-like parasite.

*B. divergens* infects cattle in Europe but has never been reported to do so in the United States. The parasite that caused the index bovine case of *B. divergens* infection, which was described in 1911 (27), is unavailable for molecular characterization. However, the DNA sequences of the complete 18S rRNA gene for bovine isolates of *B. divergens* from three European countries (6) and an isolate from an infected *Ixodes ricinus* tick from another European country (N.J. Pieniazek, unpub data) are identical; sequence

data for the complete 18S rRNA gene of *B. divergens* from its tick vector have never previously been reported.

*B. divergens* has traditionally been considered not only a bovine parasite but also the etiologic agent of most of the reported zoonotic cases of babesiosis in Europe; the cases purportedly caused by *B. divergens* typically have occurred in asplenic persons, who died if not appropriately and expeditiously treated (5). The type and quality of the evidence used to support the conclusion that *B. divergens* caused the zoonotic cases have varied markedly (5,6). To our knowledge, sequence data for the 18S rRNA gene have been reported for only two such cases. For one of the two cases (28), sequence data for the complete 18S rRNA gene were reported (EMBL data base no. AJ439713; data for 1,728 bases), which were not identical to the sequence for bovine *B. divergens* (6). For the other case (29), sequence data for a portion of the gene were reported (GenBank no. AF435415; data for 369 bases). Some of the European zoonotic cases attributed to *B. divergens* infection might have been caused by EU1, the etiologic agent of the first reported zoonotic cases of babesiosis in Italy and Austria, which occurred in 1998 and 2000, respectively (6). In phylogenetic analysis, EU1 is most closely related to *B. odocoilei* (17,19) and is secondarily related to *B. divergens* (31 base differences) (6).

Besides our case, two other U.S. zoonotic cases have been attributed to infection with *B. divergens*-like organisms, on the basis of sequence data for the 18S rRNA gene (12,13). The first, a fatal case in Missouri in 1992, occurred in a 73-year-old asplenic man, whose parasitemia level was 3%–4% (12). In the original description of the case and the etiologic agent (MO1, for "Missouri 1") (12), molecular data were provided for only a 128-bp fragment (with three unresolved positions), in which MO1 and *B. divergens* have identical sequences (6,12). The other U.S. case occurred in Kentucky in 2001, in a 56-year-old asplenic man, with a parasitemia level of 30% to 35% (13). The sequence of the complete 18S rRNA gene of the etiologic agent reportedly differs by three bases from that for *B. divergens* (similarity score, 99.8%). However,



because no GenBank accession number was provided (13), we do not know whether the three base differences constitute a subset of the eight we found between our patient's parasite and *B. divergens*.

We do not know enough about the biology of the etiologic agents of these three U.S. cases attributed (by molecular criteria) to infection with *B. divergens*–like parasites, to conclude how closely related the parasites are to the European bovine *B. divergens*. Various wild ruminants in the United States and Europe have been found to be infected with parasites that are considered *B. divergens*–like in some respects (e.g., are in the same phylogenetic clade, demonstrate serologic cross-reactivity in IFA testing, have similar morphologic characteristics [Figure 2]). Some such parasites (e.g., *B. odocoilei*, a parasite of white-tailed deer [6,17,19]; 30 base differences from *B. divergens* [similarity score, 98.3%]) are known to be different species than *B. divergens*. However, the classification by species of some *B. divergens*–like parasites remains unresolved (6). For example, the sequence for the complete 18S rRNA gene for a reindeer *Babesia* sp. in Scotland differs by only four bases from that of *B. divergens* (similarity score, 99.8%). Although the organism is not known to cause overt disease in local cattle and did not infect jirds injected with several-day-old blood from infected reindeer (30), the biologic data available to date are not definitive. Until more parasites that are *B. divergens*–like by molecular criteria, such as the parasites that caused the three U.S. zoonotic cases, are identified and characterized in other respects, we will not know whether the parasites are synonymous with *B. divergens* or belong to a complex of related species or strains.

The public health importance of the *B. divergens*–like organism in Washington is not yet known and may take years to determine. Its biology, geographic distribution, ecology, tick vector, and animal reservoir host(s), as well as the prevalence of infection in nonhuman and human hosts, risk factors for infection and disease in humans, and clinical manifestations of infection must be further investigated. We have begun our search for the tick vector; however, no ticks were found in September 2002, after flagging for 18 person-hours near the patient's house. Molecular analysis at CDC of DNA from 98 ticks of various species, from various animals and counties in Washington, showed that none were infected with the patient's parasite, but 11 were infected with *B. odocoilei* (data not shown).

The clinical aspects of our patient's case are notable, particularly the fact that he survived, despite critical illness. The extent to which host (e.g., advanced age) versus parasite factors contributed to the severity of the case are unknown. However, even *B. microti*, which traditionally has been considered less virulent than *B.*

*divergens* in humans (i.e., *B. microti* infection often is asymptomatic or associated with mild, nonspecific symptoms), has been associated with critical illness and fatalities, particularly among elderly, asplenic, or otherwise immunocompromised, patients (3,4,31,32). His remarkably good physical condition for his age and the meticulous medical care he received likely contributed to his survival.

We cannot generalize from his case to conclude what constitutes optimal antimicrobial therapy for infection with the patient's parasite. The one clinical trial in which the effectiveness of antimicrobial regimens for treatment of babesiosis was evaluated included only patients who were not severely ill and were infected with *B. microti* (33). Anecdotal data and extrapolation from the literature about treatment of malaria suggest that exchange transfusion may be beneficial for some critically ill patients, especially for those with signs of hemodynamic instability or high parasitemia levels (e.g.,  $\geq 10\%$ ) (31,34–36). The persistence of PCR positivity in our patient for at least 2 months after he completed therapy (Table 2) indicates he continued to have subpatent parasitemia, despite remaining clinically well. Persistence of PCR positivity after treatment of symptomatic cases of *B. microti* infection with clindamycin and quinine has also been reported (37,38); PCR positivity lasted a mean of 16 days in 22 such patients, about one third of whom had persistent positivity for >1 month (none for >3 months) (37).

The case we describe underscores several points for clinicians. First, the diagnosis of babesiosis should be considered for febrile persons with hemolytic anemia, regardless of where they live or have traveled. Second, babesiosis, which can be life threatening, can be caused by novel parasites not detected by serologic or molecular testing for *B. microti* or the WA1- or CA1-type parasites (i.e., for parasites previously recognized to cause zoonotic babesiosis in the United States). This fact underscores the importance of manual examination of smears of blood from patients who might have babesiosis. In most hospitals, blood smears are examined by machines rather than by laboratory staff, unless specific criteria are met by the patient, certain abnormalities are "flagged" by the machine, or manual examination is specifically requested. Third, thorough characterization of novel *Babesia* spp. is needed to advance our knowledge about zoonotic parasites and to facilitate development of laboratory methods for detecting such parasites in patients, participants in epidemiologic investigations, and ultimately, perhaps, blood donors. Characterization of novel *Babesia* spp. would be facilitated if clinicians with patients likely infected outside of the geographic areas known to be endemic for *B. microti* sent fresh, pretreatment, anticoagulated, whole blood specimens, by overnight mail,

on wet ice packs, to a reference laboratory experienced in doing such work.

### Acknowledgments

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Dr. Herwaldt is a medical epidemiologist in the Division of Parasitic Diseases of the Centers for Disease Control and Prevention. Her research interests include the clinical and epidemiologic aspects of various parasites, such as *Babesia*, *Leishmania*, *Trypanosoma*, and *Cyclospora* spp.

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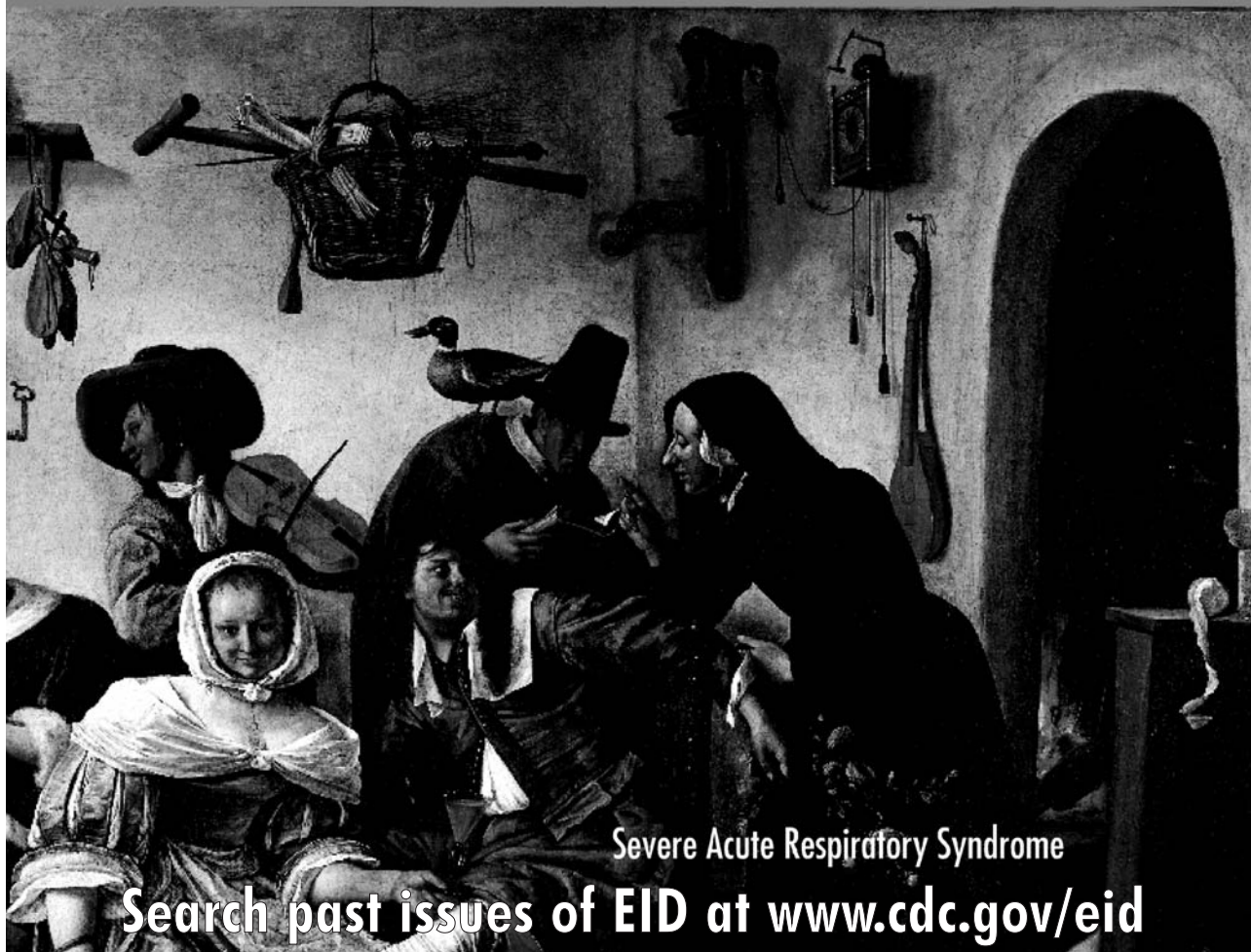
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# Influenza A Virus PB1-F2 Gene in Recent Taiwanese Isolates

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Influenza A virus contains eight RNA segments and encodes 10 viral proteins. However, an 11th protein, called PB1-F2, was found in A/Puerto Rico/8/34 (H1N1). This novel protein is translated from an alternative open reading frame (ORF) in the PB1 gene. We analyzed the PB1 gene of 42 recent influenza A isolates in Taiwan, including 24 H1N1 and 18 H3N2 strains. One H1N1 isolate and 17 H3N2 isolates contained the entire PB1-F2 ORF of 90 residues, three amino acids (aa) longer than the PB1-F2 of A/Puerto Rico/8/34 at the C terminal. The one remaining H3N2 isolate encoded a truncated PB1-F2 with 79 residues. The other 23 H1N1 isolates contained a truncated PB1-F2 of 57 aa. Phylogenetic analysis of both the HA and the PB1 genes showed that they shared similar clustering of these Taiwanese isolates, suggesting that no obvious reassortment occurred between the two genomic segments.

Influenza A virus is a prevalent pathogen with substantial pandemic potential (1). The influenza pandemic in 1918 was the most catastrophic in history, claiming more than 20 million lives (2). Influenza epidemics continue to occur every 2 to 3 years, causing substantial illness and death. Influenza A virus has eight segments of negative-stranded RNA genome and encodes 10 viral proteins (3). However, an 11th influenza A viral protein was recently found. This new protein product was discovered serendipitously during a systematic search of potential antigenic peptides recognized by CD8<sup>+</sup> T lymphocytes encoded by influenza virus A/Puerto Rico/8/34 (H1N1) (4). Unlike other influenza A viral proteins, this novel protein has several unusual features. It is absent from some influenza virus isolates, expresses different levels among individual infected cells, degrades rapidly, and localizes to mitochondria. This protein is called PB1-F2 because it is translated

from an alternative open reading frame (ORF) in the PB1 gene. The PB1 gene has an inefficient start codon, according to Kozak's analysis (5), which explains why PB1-F2 can be translated when an alternative start codon is used at nucleotide positions 119 to 121 based on the PB1 gene of A/Puerto Rico/8/34 (4).

Since PB1-F2-induced apoptosis in a cell-specific manner and might be important in pathogenesis, do the recently circulated influenza isolates possess the alternative ORF? This study analyzed 42 influenza A isolates in Taiwan from 1995 to 2001. The sequence of the hemagglutinin gene (HA) was also analyzed for further genetic characterization of these isolates (6–8).

## Materials and Methods

### Clinical Isolates

The clinical specimens examined were throat swab specimens collected from the Clinical Virology Laboratory, Chang Gung Memorial Hospital (Contract Laboratory of the Center for Disease Control and Prevention, Taiwan). The specimens were added to Madin-Darby canin kidney (MDCK) cells. Typing of the influenza A virus then was conducted by using immunofluorescence assay (IFA) by type-specific monoclonal antibody (Dako, Cambridgeshire, UK). Moreover, subtyping was conducted by reverse transcription-polymerase chain reaction (RT-PCR) with subtype-specific primers (Table) (9,10). Virus isolates were stored at –80°C until use.

### RNA Extraction and RT-PCR

The clinical isolates were passed in MDCK cells, and the supernatant was used for viral RNA extraction with the Viral RNA Extraction Miniprep System kit (Viogene, Sunnyvale, CA). Viral RNA was amplified into double-stranded DNA by RT-PCR by using the Ready-To-Go RT-PCR Beads (Amersham Biosciences, Piscataway, NJ). The Table lists the primers used for RT-PCR, and the RT-PCR

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Table. Primers used for reverse transcription–polymerase chain reaction

Primer/nt position	Sequence	Size (bp)
H1F-78 <sup>a</sup>	GATGCAGACACAATATGTATAGG	611
H1R-689 <sup>a</sup>	CICTACAGAGACATAAGCAITTT	
H3F-114 <sup>b</sup>	TCAGATTGAAGTACTAATGCT	976
H3R-1120 <sup>b</sup>	AATTTTGATGCCTGAAACCGT	
PB1F-32 <sup>c</sup>	TCAATCCGACCTTACTTTTC	409
PB1R-441 <sup>c</sup>	AGCAGGCTGGTTTCTATTTA	

<sup>a</sup>Genomic position based on A/Puerto Rico/8/34(H1N1) nc002017.<sup>b</sup>Genomic position based on A/Hong Kong/1/68(H3N2) af348176.<sup>c</sup>Genomic position based on A/Puerto Rico/8/34(H1N1) isdn13420.

program in all cases was as follows: 42°C for 30 min, 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, and a final elongation step of 72°C for 10 min. The final product was stored at 4°C.

### Nucleotide Sequence Analysis

The RT-PCR product was purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The nucleotide sequence of the purified fragments was determined by using an automated DNA sequencer. A 561- to 564-nt HA sequence was obtained for H1N1 isolates from genomic position 120 to 683 of A/Puerto Rico/8/34 (H1N1), and an 844-nt sequence was obtained for H3N2 isolates from genomic position 187 to 1031 of A/Hong Kong/1/68 (H3N2). A 300-nt PB1 sequence was obtained from genomic position 104 to 403, covering the entire PB1-F2 gene from position 119 to position 379 of A/Puerto Rico/8/34 (H1N1). Sequence analysis, including pairwise sequence alignment and protein translation, was conducted with the Lasergene software, version 3.18 (DNASTAR, Madison, WI) (11). Multiple sequence alignment was conducted with Clustal W, version 1.81 (12), with a gap opening penalty of 15 and a gap extension penalty of 6.66. The phylogenetic analysis was performed with PHYLIP (13,14), version 3.573c, with a Kimura 2-parameter distance matrix (program dnadist) and the neighbor joining method (program neighbor). Support for tree topology was determined by bootstrap analysis with 1,000 pseudo-replicate datasets generated by the seqboot program in PHYLIP. A consensus tree was obtained by the consense program, and the topology was viewed with TreeView, version 1.6.6. Secondary structures of the sequences were predicted by using the NNPREDPREDICT program (<http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html>) (15). The reference sequences were obtained from GenBank. All human influenza A viruses with H1N1 or H3N2 subtypes whose PB1 genes could be translated into a putative PB1-F2 ORF were included for comparison with the Taiwanese strains used in this study. All human influenza A viruses with other subtypes or nonhuman influenza A viruses capable of being translated into PB1-F2 were also studied.

### Nucleotide Sequence Accession Number

The nucleotide sequence data reported in this study were deposited to the GenBank nucleotide sequence database with accession numbers AY303701 to AY303752, AF139930 to AF139940, and AF362778 to AF362820.

## Results

### Influenza A isolates in the Clinical Virology Laboratory

A total of 8,229 clinical specimens were received for diagnosis of the respiratory tract virus at the Clinical Virology Laboratory, Chang Gung Memorial Hospital, from 1995 to 2001. Five hundred and forty-seven influenza A viruses were isolated from these specimens, including 170 H1N1 and 377 H3N2 isolates. Notably, no H3N2 isolates were found in 1995, and only one H1N1 isolate each was found in 1997 and 1998 (Figure 1).

### PB1 Sequence Analysis

The start codon for the PB1 gene is located at positions 25 to 27, while the stop codon is at positions 2296 to 2298, yielding a PB1 protein with 757 residues (16,17). The complete ORF for PB1-F2 in A/Puerto Rico/8/34 (H1N1) is from position 119 to 379, which encodes an 87-residue protein (4). To determine the existence of PB1-F2 in these Taiwanese influenza A isolates, 42 of them were randomly selected between 1995 and 2001, including 24 H1N1 strains and 18 H3N2 strains. Ten H1N1 and 17 H3N2 reference strains were also included for the PB1 sequence analysis. The selection was based on an extensive search for all human H1N1 and H3N2 influenza viruses in GenBank, whose PB1 nucleotide sequences were shown to alternatively translate into a PB1-F2 gene. Figures 2 and 3 show the translated PB1-F2 amino acids for H1N1 and H3N2

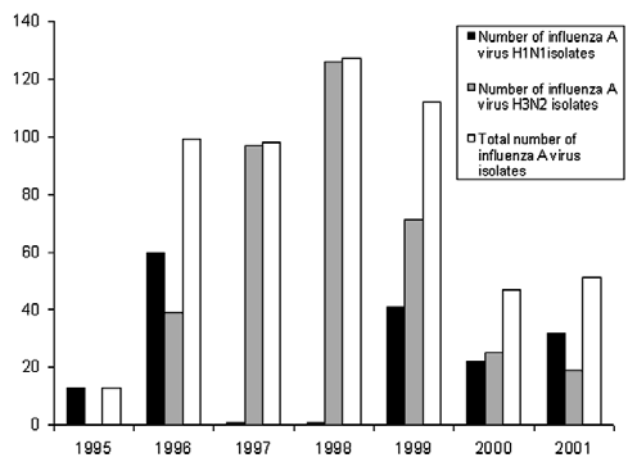


Figure 1. Number of isolated influenza A viruses in Clinical Virology Laboratory, Chang Gung Memorial Hospital, a contract laboratory of the Center for Disease Control and Prevention—Taiwan.

strains. The amino acid sequence of PB1-F2 for A/Puerto Rico/8/34 (H1N1) was included in both figures as a template. All 24 H1N1 influenza A viruses contained an alternative start codon (AUG) at positions 119 to 121 in the PB1 gene, which translated into Met (M) and marked the beginning of PB1-F2 ORF. Most of these H1N1 strains encountered a stop codon (UAG) at positions 290 to 292, resulting in the production of a 57-residue peptide and covering only a truncated PB1-F2. One isolate A/Taiwan/3355/97 (H1N1), however, covered the entire ORF (87-residue) because a non-stop codon UGG was found at equivalent positions at which other Taiwanese H1N1 encountered a stop codon. The translation continued and eventually stopped at positions 389 to 391, which encoded a putative protein of 90 residues, three residues longer than the PB1-F2 protein in A/Puerto Rico/8/34. For the nine reference strains in addition to A/Puerto Rico/8/34, three strains (A/WSN/33, A/Fort Monmouth/1/47 and A/Wisconsin/10/98) encoded a PB1-F2 of 90 residues, five strains (A/Beijing/11/56, A/Fuji/15899/83, A/Charlottesville/31/95, A/Hong Kong/470/97, and A/Hong Kong/427/98) encoded a truncated ORF of 57 residues, and one strain (A/Wisconsin/3523/88) encountered an exceptionally early stop codon and ended in only 11 residues. Most of the H3N2 isolates analyzed in this study, on the other hand, covered the full-length of PB1-F2 ORF of 90 residues as found in A/Taiwan/3355/97 (H1N1). Only two exceptions were observed, including one Taiwanese strain A/Taiwan/1748/97 with a truncated PB1-F2 ORF of 79 residues, and one reference strain A/Shiga/25/97 with 87 residues as in A/Puerto Rico/8/34 (H1N1).

The nucleotide sequence identities for PB1 gene were 96.3%–100% among the 24 Taiwanese H1N1 strains, and 97.3%–100% among the 18 H3N2 strains, based on a 300-nt segment that covers the entire putative PB1-F2 of interest. Figure 4 presents the PB1 phylogenetic tree for all 42 Taiwanese isolates and 27 previously selected reference strains. All Taiwanese strains were grouped into their respective subtype. The Taiwanese H1N1 strains were divided into two clusters. One contained eleven 1995–1996 isolates, which were similar to A/Charlottesville/31/95 and A/Hong Kong/427/98 (97.0%–98.6%), and the other contained 13 1997–2001 isolates, which were similar to A/Hong Kong/470/97 (99.0%–99.6%). The Taiwanese H3N2 strains were also divided into two clusters, including four 1996–1997 isolates similar to A/Shiga/25/97 (98.6%–99.6%) and fourteen 1997–2001 isolates similar to A/Hong Kong/497/97 and A/Hong Kong/498/97 (98.3%–100.0%).

The putative PB1-F2 protein of A/Taiwan/3355/97 (H1N1) was 81.1% identical to A/Puerto Rico/8/34 (H1N1). In addition to the three extra amino acids (Trp88-Thr89-Asn90) at the C-terminal end, A/Taiwan/3355/97

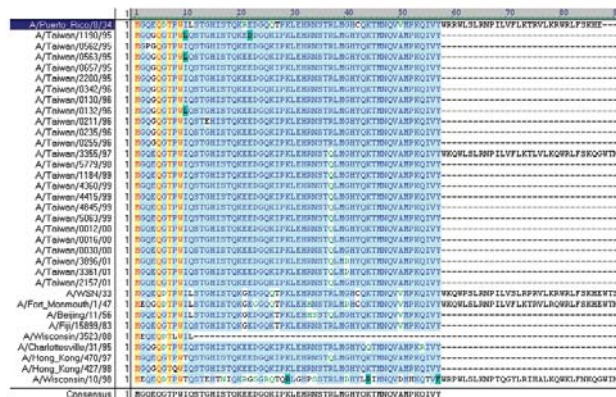


Figure 2. Alignment of putative PB1-F2 amino acid sequences of 24 Taiwanese H1N1 strains and 10 H1N1 reference strains. Most strains, including 23 Taiwanese strains and 5 reference strains, contained a truncated open reading frame (ORF) 57 residues long. One reference strain, A/Wisconsin/3523/88, had the ORF truncated at 11 residues. PB1-F2 of A/Puerto Rico/8/34 with 87 residues is placed on top of the alignment. One Taiwanese strain and three reference strains each contained a complete PB1-F2 of 90 residues. The program AlignX in VectorNTI Suite produced the alignment.

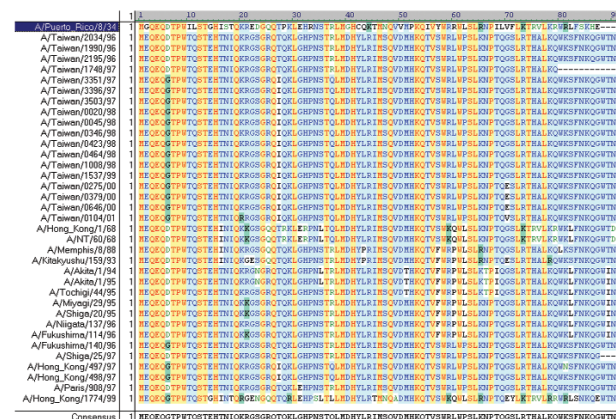


Figure 3. Alignment of putative PB1-F2 amino acid sequences of 18 Taiwanese H3N2 strains and 17 H3N2 reference strains. PB1-F2 of A/Puerto Rico/8/34 (H1N1) with 87 residues is laid over the alignment for reference. Most strains contained a PB1-F2 ORF 90 residues long. One Taiwanese strain, A/Taiwan/1748/97, encoded a truncated open reading frame (ORF) with 79 residues, and one reference strain, A/Shiga/25/97, encoded a 87-residue product as in A/Puerto Rico/8/34 (H1N1). The program AlignX in VectorNTI Suite produced the alignment.

(H1N1) had 14 aa that differed from the 87-residue peptide of A/Puerto Rico/8/34. Notably, two Arg residues, Arg75 and Arg79, which have a propensity to form an amphipathic helix and may be required for membrane translocation (4,15), had been changed to Leu75 and Gla79 in A/Taiwan/3355/97 (H1N1). However, A/Taiwan/3351/97 (H3N2) (Figure 3), a strain that also contains a 90-residue PB1-F2 and was isolated at approximately the same time as A/Taiwan/3355/97 (H1N1), was only 50.0% identical

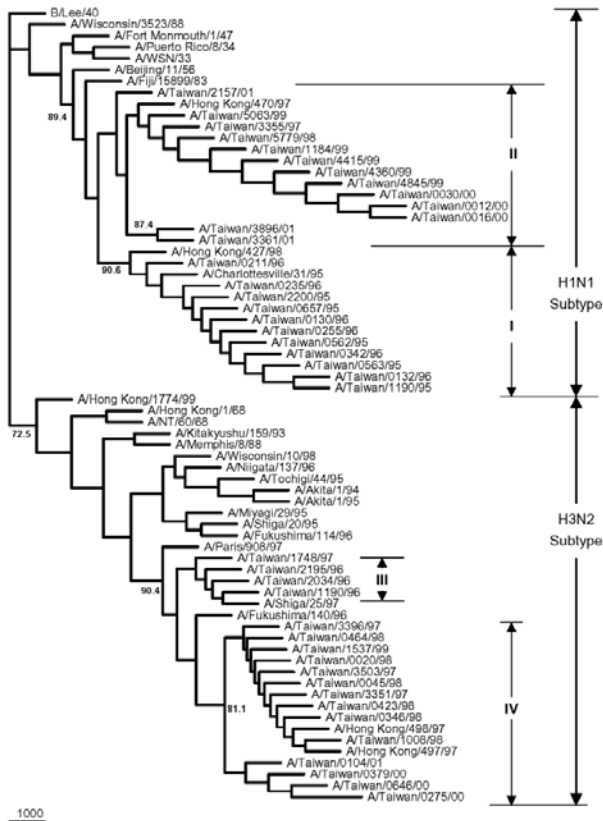


Figure 4. Phylogenetic tree of influenza A viruses for their PB1 gene nucleotide sequences. Apart from the 42 Taiwanese isolates obtained in this study, 27 reference strains were included; these were selected on the basis of an extensive search of all human H1N1 and H3N2 influenza viruses from GenBank, whose PB1 sequences were shown to be able to be translated into the putative PB1-F2 gene. The tree was rooted with B/Lee/40. All strains were separated into two groups according to their subtypes. Sequence analysis was conducted using the software Lasergene, Clustal W and PHYLIP with 1,000 replicates. All sequences are 300-nt long from genomic position 104 to 403 based on the PB1 gene of A/Puerto Rico/8/34, which covers at least the entire PB1-F2.

to A/Puerto Rico/8/34 and differed in 42 aa other than the three trailing residues. The difference between A/Taiwan/3355/97 (H1N1) and A/Taiwan/3351/97 (H3N2), both with a 90-residue PB1-F2, was 37 residues; these two strains share only 58.8% identity.

**HA Nucleotide Sequence Analysis**

The HA sequences were also analyzed to elucidate the genetic characteristics of these Taiwanese influenza A isolates. Figure 5 shows the phylogenetic tree of HA for 35 H1N1 strains, including 24 Taiwanese isolates, 6 reference strains used in earlier PB1-F2 analysis, whose HA sequences were available in GenBank, and 5 recent H1N1 vaccine strains. The sequence identity among these 24 isolates was 91.8%–100%, based on a 561- to 564-nt HA segment. Eleven 1998–2001 isolates were clustered with the

2000–2001 vaccine strain A/New Caledonia/20/99 (18–20), exhibiting a nucleotide sequence identity from 97.5% to 99.2%. Another eleven 1995–1996 isolates were clustered with A/Bayern/7/95 (21–24), the vaccine strain used in 1997–1998, and A/Charlottesville/31/95, exhibiting a nucleotide sequence identity from 97.8% to 100%. The other two Taiwanese isolates, A/Taiwan/3355/97 and A/Taiwan/1184/99, were separated from the 1998–2001 Taiwanese isolates with good bootstrap values. A/Taiwan/1184/99 was similar to A/New Caledonia/20/99, exhibiting an identity of 99.2%. A/Taiwan/3355/97, although further apart, was also similar to A/New Caledonia/20/99 too with a 97.5% identity. A/Taiwan/3355/97 was also adjacent to the preceding vaccine strain A/Beijing/262/95, as determined from phyloge-

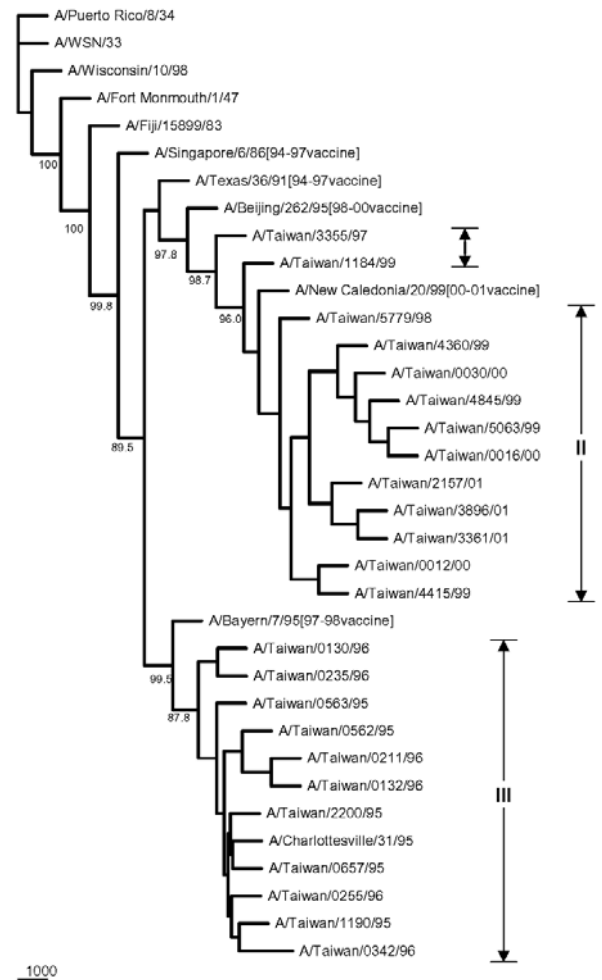


Figure 5. Phylogenetic tree of H1N1 influenza A viruses for HA nucleotide sequences. Five recent vaccine strains were included along with the 24 Taiwanese strains and six reference strains considered previously in Figure 4. The tree was rooted with A/Puerto Rico/8/34. Sequence analysis was conducted using the software Lasergene, Clustal W, and PHYLIP with 1,000 replicates. Sequences contained 561 to 564 nt bases from positions 120 to 683, based on A/Puerto Rico/8/34.



ny and shared a 96.4% identity. Nevertheless, all 13 Taiwanese H1N1 isolates from 1997 to 2001 exhibited a characteristic deletion mutation Lys (K) at aa 134 of the HA gene, as has been previously reported (25). The distinct genetic feature of possessing the entire PB1-F2 ORF in A/Taiwan/3355/97 might have separated this strain from other 23 Taiwanese H1N1 isolates in the phylogenetic tree. Figure 6 shows the phylogenetic tree of 37 H3N2 strains, including 17 Taiwanese isolates, 13 reference strains used in preceding PB1 analysis, whose HA sequences were available, 6 recent H3N2 vaccine strains, and 1 English strain as an outgroup. The nucleotide sequence identity in terms of HA gene among the 17 analyzed Taiwanese H3N2 isolates from 1996 to 2001 was 96.2%–100%, based on an 844-nt HA segment, which is higher than that among the 24 Taiwanese H1N1 isolates (91.8%–100%) under investigation. The two 1999 isolates were clustered with A/Moscow/10/99 (99.6%–99.7%), which was the vaccine strain used in 2001 to 2002 (26). The three 2000 isolates were clustered with the 2000–2001 vaccine strain A/Panama/2007/99 (98.8%–99.4%) (18). The eight 1997–1998 isolates formed their own cluster, despite a relatively low bootstrap support, and were similar to the 1998–2000 vaccine strain A/Sydney/5/97 (98.8%–99.4%) (24,27,28). Four other Taiwanese 1996–1997 isolates were not grouped into any of these Taiwanese H3N2 clusters. They were similar to A/Shiga/25/97 and the two 1996 Fukushima strains (97.5%–99.5%).

## Discussion

One isolate among the 24 Taiwanese H1N1 strains from 1996 to 2001 possessed the full-length PB1-F2 ORF. Most of the H1N1 isolates contained a shorter 57-residue putative PB1-F2, encountering a premature stop codon at positions 290 to 292. All of the Taiwanese H3N2 isolates, however, contained the full-length ORF from 1995 to 2001, except for A/Taiwan/1748/97, which encoded a truncated PB1-F2 of 79 residues. The putative full-length PB1-F2 of these Taiwanese strains contained 90 aa, which was three residues longer than A/Puerto Rico/8/34 (H1N1).

PB1-F2 of A/Puerto Rico/8/34 (H1N1) has been shown to localize to the mitochondria and induce cell death. According to the NNPREDPREDICT algorithm, PB1-F2 of A/Puerto Rico/8/34 (H1N1) tends to form an amphipathic helix, extending from Leu69 to Phe83 (4,15). The predicted helix includes five basic residues. This feature enables PB1-F2 to translocate through the membrane, target the mitochondria, and trigger host cell apoptosis (29). The putative 90-residue-long PB1-F2 protein of A/Taiwan/3355 (H1N1) and A/Taiwan/3351 (H3N2) also possessed a predicted helix from Leu72 to Phe83. However, A/Taiwan/3355/97 (H1N1) contained three basic residues, and A/Taiwan/3351/97 (H3N2) contained four.

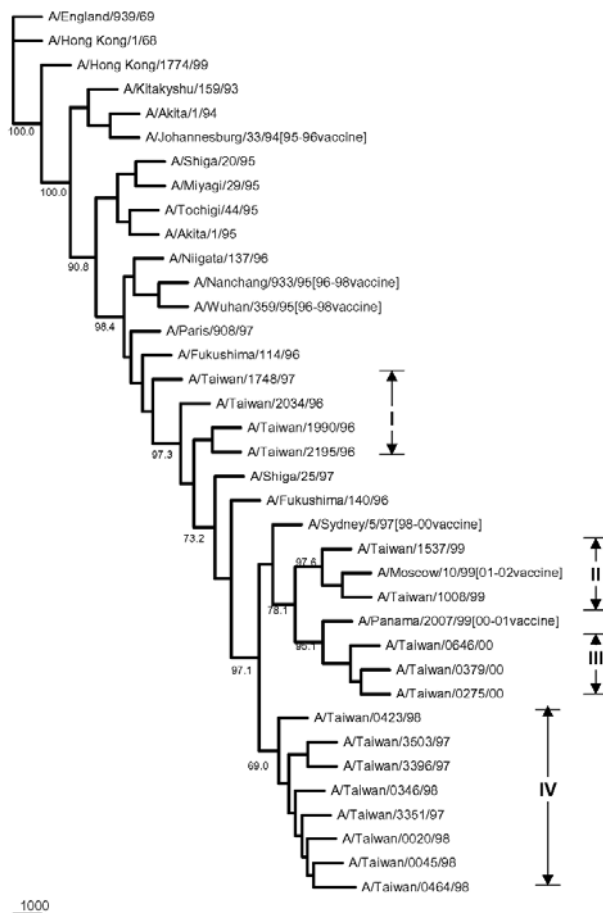


Figure 6. Phylogenetic tree of influenza A H3N2 viruses for HA nucleotide sequences. Six recent vaccine strains were included along with the 17 Taiwanese strains and 13 reference strains shown in Figure 4. The tree was rooted with A/England/939/69. Sequence analysis was conducted by using the software Lasergene, Clustal W, and PHYLIP with 1,000 replicates. All sequences are 844-nt long from positions 187 to 1031, based on A/Hong Kong/1/68.

The truncated PB1-F2 (57-residue ORF of most Taiwanese H1N1 strains or the 79-residue ORF of A/Taiwan/1748/97 [H3N2]) did not have the predicted helix. The change in the number of basic residues in those Taiwanese strains did not alter the result from NNPREDPREDICT, that is, all full-length PF1-F2 amino acid sequences (87- or 90-residue ones) were predicted to contain a helix around the same location. Whether the reduction in the number of basic residues in the predicted helix, or the significant difference between the PF1-F2 amino acids of the Taiwanese strains and of A/Puerto Rico/8/34 (H1N1), supports functions that differ from those of A/Puerto Rico/8/34 (H1N1), remains to be investigated.

The truncated PB1-F2 (with <87 or 90 aa residues) did not contain the mitochondrial translocation signal at the



C-terminal end and may lose the PF1-F2 functions described above. The earliest observation for such truncation of human H1N1 or H3N2 influenza A viruses was associated with A/Beijing/11/56 (H1N1), which has been seen in most Taiwanese H1N1 strains and four other reference strains (Figure 3), as well as in A/Taiwan/1748/98 (H3N2) (Figure 4).

All of the H1N1 Taiwanese isolates from 1997 to 2001 exhibited a stable genetic characteristic—a deletion mutation at aa 134 in the HA gene. The only H1N1 isolate in 1997, A/Taiwan/3355/97, was found to encode the entire 90-residue PB1-F2 ORF, which is 3 aa longer than the PB1-F2 of A/Puerto Rico/8/34 (H1N1). Although A/Taiwan/3355/97 (H1N1) was found to be phylogenetically related to those isolates that encode partial PB1-F2 from 1998 to 2001 in both HA and PB1 genes, this genetic marker that putatively encodes a full-length PB1-F2 was not retained in subsequent isolates. This finding suggests that the existence of a putative full-length PB1-F2 ORF in the PB1 gene might have been a disadvantage to the growth of H1N1 virus in the infected host population. However, almost all of the H3N2 isolates analyzed in this study encoded a full-length ORF of PB1-F2, including the Taiwanese strains and the reference strains from GenBank. The only exception was A/Taiwan/1748/97, which encoded a truncated PF1-F2 with 79 residues.

The 336 influenza A PB1 sequences of all species from GenBank, including all the Taiwanese and reference strains analyzed in this work, were collected to provide an overall picture of how a putative PB1-F2 can be alternatively translated from its PB1 gene. All 336 PB1 sequences examined contained the entire RNA segment equivalent to the position of PB1-F2 ORF as in A/Puerto Rico/8/34 (H1N1). Only two avian strains without a start codon were observed (A/Quail/Hong Kong/AF157/92 [H9N2] and A/duck/NC/91347/01 [H1N2]), leaving no PB1-F2 encoded. Ninety-nine human influenza A viruses were observed, among which 64 encoded a 90-residue PB1-F2, two encoded an 87-residue ORF (A/Puerto Rico/8/34 [H1N1] and A/Shiga/25/97 [H3N2] stated early section), and 33 encoded a truncated ORF (1 strain with 79 residues, 30 with 57, and 2 with 11). Of the remaining 235 nonhuman influenza A viruses, 191 encoded a 90-residue PB1-F2, 7 encoded an 87-residue ORF, and 37 encoded a truncated ORF. (Twenty-three strains had 79 residues, 2 had 63; 5 had 57; 1 had 34, and 6 had 11.) Of all the 334 translated PB1-F2, 264 (79.0%) contained a complete ORF of 87 or 90 residues.

The complete PB1 sequences of 38 influenza A viruses, including 26 human and 12 nonhuman strains, were gathered from GenBank to evaluate the genetic variation of the PB1 gene and the putative PB1-F2 gene. Each sequence is 2,271 to 2,274-bp long, covering the entire coding region

of PB1 and containing a putative PB1-F2 ORF of at least 87 residues. The pairwise identities of these 38 PB1 coding sequences were from 81.2% to 99.9% for nucleotides, and 94.3%–99.7% for the translated amino acids. For the alternatively translated PB1-F2 ORF that is 261- to 270-bp long, or from nucleotide position 95 to 364 with respect to the full coding region of PB1, the pairwise identities were 79.2%–99.6% for nucleotides, and 52.2%–98.8% for the translated amino acids. In terms of the partial sequences in the forms of their regularly translated PB1 segments (from position 94 to 363, or a 70-residue peptide) equivalent to a genomic range in which the PB1-F2 is translated, the pairwise identities for these 38 strains were 92.2%–100.0%. The variation among the sequences was limited to approximately 20% in nucleotides and <8% in amino acids for the complete PB1 coding region, as well as for a partial PB1 segment (position 94 to 363) corresponding to the PB1-F2 location. The alternatively translated PB1-F2 to the equivalent piece of RNA (position 95 to 364), however, exhibited a large variation up to 50%. The two translations differed by only 1 nt. Although certain single nucleotide mutations, which might have occurred at the third position of a codon in the original PB1 translation, did not seem to substantially alter the resulting amino acid sequences, a frame shift of alternatively translating the RNA into a putative PB1-F2 apparently introduced a substantial change in the genetic heterogeneity up to 47.8%. A single nucleotide change can also easily introduce a stop codon under this new translation, which might account for the observed truncated PB1-F2 genes of various lengths. PB1-F2 protein is not essential to influenza viral replication, so this novel gene may be subject to less selection pressure than PB1 or HA during viral evolution.

Phylogenetic inference drawn from PB1 sequences showed two distinct clusters within the H1N1 and H3N2 groups of the analyzed Taiwanese strains (Figure 4). Twenty-four Taiwanese H1N1 strains were grouped into cluster I (which contained eleven 1995–1996 strains) and cluster II (which contained thirteen 1997–2001 strains). This same clustering pattern was seen in the HA phylogenetic tree for H1N1 strains in Figure 5, in which the eleven 1995–1996 strains joined cluster III, and the thirteen 1997–2001 strains were in cluster I+II. A similar clustering pattern of 18 Taiwanese H3N2 strains was also observed between Figures 4 and 6. Phylogenetic analysis of both PB1 and HA genes showed no evidence of reassortment for the two viral segments among these Taiwanese isolates.

Apoptosis is an important pathogenic mechanism in influenza A virus infection. Differential induction of apoptosis was caused in the infected cells by different influenza virus strains (30). PB1-F2 is a mitochondrial protein that induces apoptosis. PB1-F2 was absent from certain

influenza A isolates, so further investigating the correlation between the existence of PB1-F2 and the apoptosis level of influenza A virus-infected cells would be of interest.

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# Coccidioidomycosis among Workers at an Archeological Site, Northeastern Utah

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In 2001, an outbreak of acute respiratory disease occurred among persons working at a Native American archeological site at Dinosaur National Monument in northeastern Utah. Epidemiologic and environmental investigations were undertaken to determine the cause of the outbreak. A clinical case was defined by the presence of at least two of the following symptoms: self-reported fever, shortness of breath, or cough. Ten workers met the clinical case definition; 9 had serologic confirmation of coccidioidomycosis, and 8 were hospitalized. All 10 were present during sifting of dirt through screens on June 19; symptoms began 9–12 days later (median 10). Coccidioidomycosis also developed in a worker at the site in September 2001. A serosurvey among 40 other Dinosaur National Monument workers did not find serologic evidence of recent infection. This outbreak documents a new endemic focus of coccidioidomycosis, which extends northward its known geographic distribution in Utah by approximately 200 miles.

Coccidioidomycosis results from inhaling spores (arthroconidia) of *Coccidioides immitis*, a soil-dwelling fungus endemic to the southwestern United States and parts of Mexico, Central America, and South America. The organism is recovered from soil in areas with yearly annual rainfall averaging 5–20 inches, hot summers, infrequent winter freezes, and alkaline soil (1). Although up to 100,000 new infections may occur annually (2), reported point source outbreaks are infrequent and have followed diverse soil-disrupting activities or events, such as archeological or anthropological digs (3–5), military maneuvers (6), play involving throwing dirt (7), construction work (8), earthquakes (9), dust storms (10,11), model airplane flying competitions (12), and armadillo hunting (13).

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We report a point-source outbreak of coccidioidomycosis among workers who participated in soil-disrupting activities at an archeological site in Dinosaur National Monument in northeastern Utah during June and July 2001. This site was approximately 200 miles north of previously known *C. immitis*-endemic areas for in Utah (Figure 1). In addition, we report results of a serologic survey of Dinosaur National Monument employees performed to assess recent exposure to *C. immitis*.

## Outbreak Setting

Dinosaur National Monument covers 320 square miles in the Uinta Basin in northeastern Utah and northwestern Colorado (Figure 1). A total of 397,800 visitors were recorded in 2000. During summer 2001, 49 permanent and 49 seasonal employees, as well as approximately 120 volunteers, worked at the monument. A weather station,

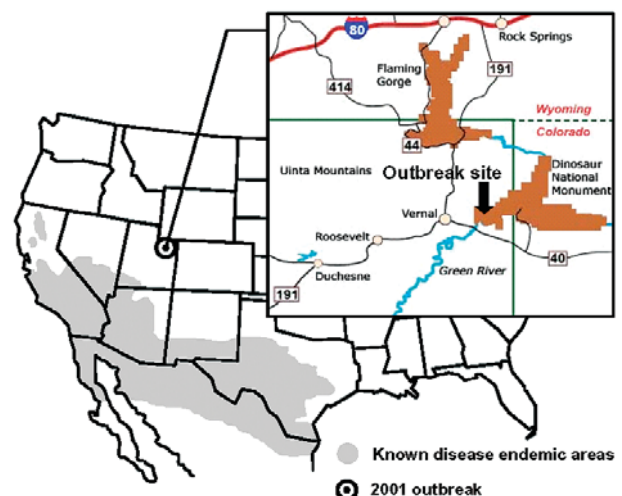


Figure 1. Known geographic distribution of *Coccidioides immitis* in the United States and location of the 2001 coccidioidomycosis outbreak in Utah. Source: U.S. Geological Survey, Operational Guidelines for Geological Fieldwork in Areas Endemic for Coccidioidomycosis (Valley fever), 2000.

located approximately 0.5 miles from the outbreak site, has recorded an average annual precipitation of 8.7 inches since 1958.

The outbreak site, at an elevation of 4,825 feet in an arid, treeless region with small hills and rock outcroppings, is under a rock overhang. The overhang faces directly south and receives reflected heat from the surrounding frontier sandstone. Heat trapped within the shelter raises the temperature several degrees above the outside temperature, hence its name, Swelter Shelter (14). Swelter Shelter's soil is a fine-grained, sandy loam containing approximately 10% clay, 10% silt, and 80% fine sand. The soil has a low water-holding capacity, an organic matter content of <5%, a pH of 8.5 to 11.0, and salinity of 8 to 16 mmhos/cm (15). Swelter Shelter is on the main automobile tour through Dinosaur National Monument and is accessed by a short trail.

Archeological excavations conducted at Swelter Shelter in 1964 and 1965 were part of a larger archeological survey of the monument that included many Native American sites (14). The inside wall of Swelter Shelter contains Native American petroglyphs and pictographs dating from the Fremont Culture before 1200 A.D. The 1964 and 1965 excavations identified artifacts as old as 7000 to 6000 B.C., as well as two ancient fireplace hearths, one of which contained burned animal bones (14). Unknown to those working in 2001, an outbreak of respiratory illness had occurred among those conducting the earlier archeological excavations (16).

On June 18, 2001, under the direction of National Park Service archeologists, a team of six student volunteers and two volunteer leaders began work at Swelter Shelter. Work included laying stone steps, building a retaining wall, and sifting dirt for artifacts—an activity that created considerable dust. Within the week before work began, the volunteers and leaders had arrived from their residences throughout the United States; one arrived from Europe. While at the monument, they camped in tents approximately 3 miles away from Swelter Shelter. During June 29 to July 3, all six volunteers, both leaders, and two National Park Service archeologists who worked at the site sought medical care at a local hospital emergency room for acute respiratory and systemic symptoms.

## Methods

### Case Definitions

Persons working at Dinosaur National Monument were defined as meeting the clinical case definition for coccidioidomycosis if they had onset after June 18, 2001, of at least two of the following symptoms: self-reported fever, difficulty breathing, and cough. Persons meeting the clinical case definition were considered to have had laboratory-

confirmed coccidioidomycosis if a complement fixation (CF) antibody titer of  $\geq 1:2$  was present or if either of the immunodiffusion tests showed a band of identity. Further confirmation of infection was obtained if there was seroconversion or a  $\geq 4$ -fold rise in antibody titer between paired serum samples.

### Cohort Study

From July 2 to 4, 2001, 18 people (all six student volunteers, both volunteer leaders, and all 10 National Park Service archaeologists at Dinosaur National Monument) were interviewed by using a standardized questionnaire to determine symptoms and activities from June 18 to 29. In addition, clinical information was gathered from emergency room and hospital records of persons who sought medical care and recorded on another standardized form. Differences in categorical variables were assessed with the Fisher exact test.

### Laboratory Studies

Acute-phase serum samples were obtained on July 1 or July 3 from persons meeting the clinical case definition and were tested for antibodies to *Francisella tularensis*, *Yersinia pestis*, *Mycoplasma* species, *Histoplasma capsulatum*, and *C. immitis* by using standard techniques at laboratories at the Centers for Disease Control and Prevention. In addition, all persons had serologic tests for *Rickettsia rickettsii*, five for *Legionella*, and five for hantavirus at local laboratories. Blood cultures for bacterial pathogens were obtained during hospitalization.

Convalescent-phase serum samples were obtained from July 16 to 21, 2001. Acute- and convalescent-phase serum samples were assayed for antibodies to *C. immitis* by CF and immunodiffusion (IDCF), primarily to detect immunoglobulin (Ig) G antibodies. Acute-phase serum samples were assayed by immunodiffusion (IDTP) primarily to detect IgM antibodies (IDTP) (17); the IDTP assay was further performed with serum concentrated three- to fivefold.

### Environmental Investigation

Monthly average temperature and precipitation data for the Dinosaur National Monument quarry area (approximately 0.5 miles from Swelter Shelter) for 1958 to 2002 were obtained from the Western Regional Climate Center (available from: [www.wrcc.dri.edu/cgi-bin/cliMONtavt.pl?utdino](http://www.wrcc.dri.edu/cgi-bin/cliMONtavt.pl?utdino) and [www.wrcc.dri.edu/cgi-bin/cliMONtpre.pl?utdino](http://www.wrcc.dri.edu/cgi-bin/cliMONtpre.pl?utdino)). Daily rainfall data were obtained from weather station records at the monument.

### Serologic Survey of Monument Workers

From August 15 to 17, 2001, we conducted a serologic survey among Dinosaur National Monument employees to



determine the presence of antibodies to *C. immitis*. Because coccidioidomycosis skin test reagents are currently unavailable, testing for antibodies to coccidioidomycosis was performed to assess prior immunity to *C. immitis* among persons who reside or work in the area. Samples were tested using CF and IDCF as described earlier.

## Results

### Cohort Study

Ten of the 18 persons interviewed met the clinical case definition for coccidioidomycosis. The case-patients included all 6 volunteers, both group leaders, and 2 of 10 archeologists. The median age of patients was 17 years; five were male; and all were Caucasian. Illness onsets ranged from June 28 to July 1 (Figure 2).

Because the two group leaders and six volunteers traveled as a group and all became ill, the sites of possible exposure to coccidioidomycosis were limited to Swelter Shelter and their camping area, the only two places visited before June 27. All eight of these persons reported engaging in the same activities at each site; thus, determining specific risk factors at Swelter Shelter or the camping area was not possible. However, among the 10 archeologists, 2 of 3 who worked at Swelter Shelter in June met the clinical case definition compared to none of 7 who did not work there ( $p = 0.07$ ). The two ill archeologists worked on June 19, when dirt near the petroglyphs was sifted with screens (archeologists A and B, Figure 2). The archeologist who remained healthy (archeologist C, Figure 2) only worked on June 20. On that day, sifting occurred along the trail approximately 15 feet from the petroglyph panel. Sifting did not occur on other days. Therefore, all persons meeting the clinical case definition, and none of the noncase-patients were present at the sifting on June 19 (Figure 2) ( $p = 0.00002$ ). No archeologist had visited the camping area.

With June 19 being the most likely time of exposure, the median incubation period for the 10 persons who met the clinical case definition was 10 days (range 9–12). These persons reported difficulty breathing (10 persons), nonproductive cough (9 persons), fever (9 persons), fatigue (8 persons), shortness of breath (7 persons), myalgia (6 persons), skin rash (6 persons), and nausea/vomiting (4 persons). Eight persons were hospitalized; the one person who did not report fever had a temperature of 37.8°C on hospital admission. The mean temperature on admission was 38.3°C (range 36.9°C–39.4°C) and the average respiratory rate was 23 per minute (range 18–32). Results of a pulmonary examination were relatively unremarkable except for dry cough. At the time of evaluation, five patients had a maculopapular rash on the neck, trunk, and extremities. The mean leukocyte count at admission was 11,800 mm<sup>3</sup> (range 5,600–17,700), with an average of

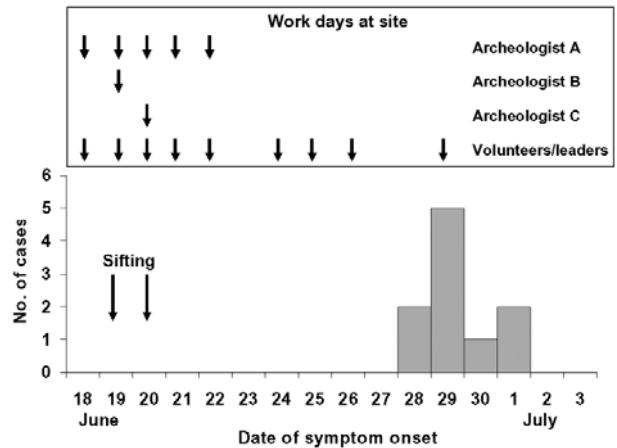


Figure 2. Number of persons meeting the clinical case definition, by date of symptom onset. Days worked at the site are indicated.

80% neutrophils (range 67%–92%). Results of tests of liver and renal function, including urinalysis, were within normal limits. The average oxygen saturation was 93% (range 88%–97%) by pulse oximetry. Chest radiographs of all 10 case-patients showed bilateral patchy infiltrates. All persons hospitalized were treated with fluconazole and discharged within 3 days.

Acute-phase serum specimens from 9 of 10 persons who met the clinical case definition contained IgM antibodies to *C. immitis*, as determined by IDTP by using concentrated serum samples; one was positive by IDTP before serum concentration. The patient without demonstrable IgM antibodies on convalescent-phase serologic testing had pulmonary infiltrates and a skin rash typical of the other patients. Two of the eight patients with convalescent-phase samples had at least a fourfold increase in CF titer. Initial serologic tests for antibodies to *F. tularensis*, *Y. pestis*, *Mycoplasma* species, *R. rickettsii*, *Legionella*, and hantavirus were negative. Blood cultures were negative for bacterial pathogens.

### Additional Case Investigation

On August 24, state and local health departments jointly recommended that employees minimize soil disturbance and dust inhalation (e.g., by watering down the soil and wearing National Institute for Occupational Safety and Health [NIOSH]-approved N95 respirators) at Swelter Shelter to reduce their risk for *C. immitis* infection. Five persons completed work on the retaining wall and steps on September 24 and 27, 2001. Although the soil was watered periodically during both days, the workers only wore masks on September 24. On October 5, 2001, left shoulder and chest pain, shortness of breath, fever, headache, diaphoresis, and shaking chills without skin rash developed in one worker. A chest radiograph on October 16

indicated left lung pneumonia. Serum samples drawn on October 15 and November 7 demonstrated elevated and rising *C. immitis* IgG and IgM antibody levels by enzyme-linked immunosorbent assays at a commercial diagnostic laboratory, a finding consistent with acute coccidioidomycosis.

### Environmental Investigation

Weather data indicated an unusually long dry period before the 2001 outbreak. No rain fell from May 3 to June 25. For May and June 1958 to 2002, the average temperatures were 14.9°C (standard deviation 1.4) and 20.1°C (standard deviation 1.6), respectively. The average temperatures in May and June 2001 were 17.1°C and 22.5°C. Since 1958, only 1 year (1994) had a warmer average temperature in both May and June.

### Serologic Survey of Monument Workers

Forty employees were enrolled. Their mean age was 43 years; 26 (65%) were male. They had worked at Dinosaur National Monument for a median duration of 26 months (interquartile range 9–126 months) and had spent an average of 13.8 years living within 100 miles of the monument. Employee activities included digging up soil or rock (27.5%), sifting sand or dirt (7.5%), and preparing paths or trails (12.5%). A quarter (27.5%) reported work duties that exposed them to dust every day. Serologic tests for *C. immitis* antibodies were negative for all 40 persons.

### Investigation of the 1964–1965 Archeological Team

To investigate the possibility that a similar outbreak occurred during the 1964–1965 archeological excavations at the monument, we interviewed by telephone the leaders of that 25-person, archeological team in January 2003. They identified five team members in whom acute respiratory illness occurred in 1964 to 1965. These five were contacted by telephone; all reported fever, dry cough, and chest pain with onsets toward the end of, or shortly after, the archeological activities. One was hospitalized for 10 days with pulmonary infiltrates; a skin rash and severe arthralgias also developed in this person. Three persons reported subsequent positive skin or serologic tests for *C. immitis*; none had previously lived in an area endemic for coccidioidomycosis. All five ill persons worked at Swelter Shelter in August 1964 or June and July 1965. None of those working at other sites had known illness.

### Conclusion

This outbreak provides evidence of an endemic focus of coccidioidomycosis within Dinosaur National Monument in northeastern Utah, expanding the known geographic distribution of coccidioidomycosis. Multiple factors likely contributed to this outbreak: most patients

lived in areas where coccidioidomycosis was not endemic and probably were without preexisting immunity to *C. immitis*; the alkalinity of the soil was favorable to the growth of *C. immitis*; a prolonged dry period occurred before the outbreak; and the archeological activities, particularly the sifting of the very fine-grained soil, caused considerable dust exposure.

Several lines of evidence indicate that the endemic focus at Swelter Shelter is highly localized and does not represent a larger unrecognized region of activity in northeastern Utah. First, the serologic survey failed to find recent serologic evidence of *C. immitis* exposure among other Dinosaur National Monument workers, although many reported frequent occupational exposure to soil or dust. However, the serologic survey could not rule out prior exposures since serologic tests are insensitive for past infection (18). Second, locally acquired coccidioidomycosis has not been reported among other monument workers or in local residents. Third, although the cause of the 1964–1965 outbreak cannot be definitively determined, the illnesses were remarkably similar to those in 2001 and were only recognized in persons working at Swelter Shelter, despite extensive archeological activities at many other sites (14).

Consistent with the hypothesis of a highly focal endemic area at Swelter Shelter, a study of outdoor workers in California indicated that even in disease-endemic areas, highly focal areas of high risk for coccidioidomycosis exist and persist (19). Another study showed that after burial of animals that died of coccidioidomycosis in soil free of the organism, repeated isolations of *C. immitis* from the soil at the burial site were possible for at least 7 years, even though soil immediately surrounding the burial site remained uncontaminated (20).

The endemic focus at Swelter Shelter may have been established centuries ago. This outbreak and the three that occurred at Native American archeological sites in California outside the known range for *C. immitis* (3–5) suggest that these sites may represent a unique type of extremely focal endemic area. *C. immitis* is highly concentrated in Native American middens, indicating that the organism was highly prevalent among these people (21), and repeated isolations of *C. immitis* have been made at old Native American campsites in endemic areas (22). Analysis of the microsatellite loci of *C. immitis* to study the genetic diversity of the fungus has shown that isolates from North America are geographically partitioned. This finding suggests that *C. immitis* was spread throughout the Americas by migrating populations of Native Americans (23); the occurrence of outbreaks at Native American archeological sites outside known disease-endemic areas supports this hypothesis. The last identified Native American presence at Swelter Shelter dated before 1200 A.D. (14).

Why *C. immitis* can apparently persist at Swelter Shelter, an area well outside the known disease-endemic region, is not known. One possibility is that average soil temperatures within the shelter are higher than surrounding areas. Swelter Shelter is the only known Native American site at Dinosaur National Monument that faces south, is relatively sheltered, and receives considerable reflected heat from surrounding rock.

Attack rates during outbreaks at Native American sites outside of disease-endemic areas have been high. During the three California outbreaks, attack rates ranged from 48% to 77% (3–5), compared to 91% among those who worked at Swelter Shelter. At the shelter, sifting of dirt with screens appeared to place persons at a particularly high risk; the 91% attack rate in this outbreak probably reflects the fact that all of the workers participated in or close to the sifting.

We conclude that a newly identified endemic focus for *C. immitis* exists at a Native American archeological site in northeastern Utah. Archeologists working at Native American sites in the western United States are at risk for coccidioidomycosis, even outside recognized disease-endemic regions. Sifting of dirt during archeological activities may put persons at a particularly high risk for exposure to *C. immitis*. Measures to decrease dust and aerosolization of spores, such as soil wetting, and the use of appropriate masks while performing archeological work at these sites may reduce the risk. Archeological workers might benefit from vaccination; efforts are currently focused on developing a vaccine for coccidioidomycosis (24)

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# Maternal Malaria and Perinatal HIV Transmission, Western Kenya<sup>1,2</sup>

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To determine whether maternal placental malaria is associated with an increased risk for perinatal mother-to-child HIV transmission (MTCT), we studied HIV-positive women in western Kenya. We enrolled 512 mother-infant pairs; 128 (25.0%) women had placental malaria, and 102 (19.9%) infants acquired HIV perinatally. Log<sub>10</sub> HIV viral load and episiotomy or perineal tear were associated with increased perinatal HIV transmission, whereas low-density placental malaria (<10,000 parasites/μL) was associated with reduced risk (adjusted relative risk [ARR] 0.4). Among women dually infected with malaria and HIV, high-density placental malaria (≥10,000 parasites/μL) was associated with increased risk for perinatal MTCT (ARR 2.0), compared to low-density malaria. The interaction between placental malaria and MTCT appears to be variable and complex: placental malaria that is controlled at low density may cause an increase in broad-based immune responses that protect against MTCT; uncontrolled, high-density malaria may simultaneously disrupt placental architecture and generate substantial antigen stimulus to HIV replication and increase risk for MTCT.

**M**alaria during pregnancy is a serious problem in sub-Saharan Africa, affecting an estimated 24 million pregnant women; malaria prevalence may exceed 50% among primigravid and secundigravid women in malaria-endemic areas (1). During the past 2 decades, HIV and AIDS have emerged as major problems in many malaria-endemic areas of sub-Saharan Africa, where an estimated 28 million persons are infected with HIV (2). Sub-Saharan Africa accounts for more than two thirds of the world's 40 million HIV-infected persons and 80% of the world's HIV-infected women, with HIV prevalence rates sometimes exceeding 40% among pregnant women (3). Without inter-

vention, as many as 30%–45% of pregnant women infected with HIV will pass the virus to their children through mother-to-child transmission (MTCT) (4), of which 15%–30% is intrauterine or intrapartum.

Given the wide overlap between areas where HIV and malaria are each prevalent, the epidemic of HIV/AIDS in areas where *Plasmodium falciparum* is endemic has generated concern about potential interactions between the two infections, especially in sub-Saharan Africa (5–9). Studies have shown increased HIV replication both in blood mononuclear cells exposed to malaria antigens in vitro (10) and in transgenic mice infected with *P. chabaudi* (11). Proviral loads are also higher among HIV-infected persons with clinical malaria compared to those without malaria; these levels remain high for at least 4 weeks after treatment (12). Although malaria may increase viral replication in the short term, the concern that the malaria-associated increase in viral replication may accelerate HIV disease progression has not been proven (13).

Studies among pregnant women in sub-Saharan Africa have provided the first evidence of an important public health problem arising from the interaction of HIV and malaria. HIV infection appears to impair malarial immunity among pregnant women, as pregnant women infected with HIV demonstrate more frequent and higher density parasitemia than pregnant women not infected with HIV (5–7,14). More recent case-control (15) and longitudinal (16,17) studies on the clinical pattern of malaria in HIV-infected, nonpregnant women have shown HIV infection to be associated with an increased frequency of clinical malaria and parasitemia, particularly among persons with

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advanced HIV disease. Data from Malawi (18) have suggested that infants exposed in utero to both placental malaria and maternal HIV infection have an increased risk for postneonatal death three- to eightfold higher than infants born to mothers with either infection alone. We examined the relationship between placental malaria and perinatal HIV transmission in an area of western Kenya with high prevalence of malaria and HIV infection.

## Participants and Methods

### Study Site

This study was conducted at Nyanza Provincial General Hospital (NPGH), a large publicly funded hospital in Kisumu (population 300,000) in western Kenya. Malaria transmission within Kisumu town is perennial, and *P. falciparum* is the predominant species, accounting for 98% of malaria cases (19). Chloroquine resistance is prevalent in the area: 75%–80% of *P. falciparum* strains show a RII/RIII resistance pattern (20). The prevalence of HIV infection among pregnant women is approximately 25% (6,21).

### Recruitment and Follow-up

Pregnant women were enrolled from June 1996 through May 2000. Screening procedures have been described previously (21). Briefly, women at the antenatal clinic were eligible for participation if they had an uncomplicated singleton pregnancy of  $\geq 32$  weeks' gestation (based on the fundal height estimation), resided within the Kisumu municipality, and had no known underlying chronic illness. Following informed consent, a structured questionnaire was administered in the local language (*Dholuo* or *Kiswahili*) to obtain information on sociodemographic, health, and obstetric factors.

A trained HIV counselor then counseled each woman, and a posttest counseling appointment was made. A blood sample was taken for HIV antibody testing, hemoglobin level, and malaria thick blood film. All screened women were encouraged to deliver at NPGH. In addition, non-screened women who delivered at NGPH were eligible for participation if they met study inclusion criteria. Routine use of zidovudine or nevirapine was not the Kenyan Ministry of Health policy during the study period, and these drugs were not available in Ministry of Health facilities.

At delivery, information was collected on mode and outcome of delivery and any illness and treatment in the previous 2 weeks. Within 24 hours of birth, infants were weighed ( $\pm 1$  g) on an electronic balance (Ohaus, Florham Park, NJ), and gestational age was assessed by using the Ballard method (22). All live, singleton, vaginally delivered infants of HIV-positive mothers were eligible for study. Infants were seen monthly until 12 months of age.

Informed consent was obtained from all women before they were enrolled in this study. Human subjects guidelines of the Centers for Disease Control and Prevention (CDC) and the Kenya Medical Research Institute ethical review committee were strictly followed. Mothers of enrolled infants signed an additional informed consent form for infant participation.

### Ethical Review

The study protocol was approved in 1995 by the institutional review boards of the Kenya Medical Research Institute; CDC, Atlanta, Georgia, USA; and the Academic Medical Center (AMC), University of Amsterdam, Amsterdam, The Netherlands, and was reviewed annually by the participating institutions. This study occurred during a changing environment in preventing perinatal HIV transmission, with results of studies in other countries demonstrating the benefits of short-course AZT use (23,24) or nevirapine use (25) in late pregnancy and perinatally to reduce the risk for MTCT. Similarly, studies of malaria prevention in pregnancy during the study interval were demonstrating the benefit of intermittent antimalarial treatment to prevent maternal anemia and low birth weight (6,26). The participating institutions and the Kenyan Ministry of Health were engaged in discussions about these findings regarding the ethical considerations for this investigation. The study continued with full ethical approvals during these discussions. Investigators and institutions have supported the transition to a system that supports providing intermittent preventive antimalarial therapy during pregnancy, according to the adopted Kenyan national policy. The MTCT prevention program now offers HIV counseling and testing, community education, and antiretroviral drugs to prevent MTCT in the study setting (4).

### Blood Sampling and Laboratory Procedures

At delivery, maternal peripheral and placental thick blood films were prepared, stained with 10% Giemsa, and examined under oil immersion for malaria parasites. Placental blood was obtained by cutting into the maternal side of the placenta and placing collected blood on a slide. A thick film was considered negative if 100 microscopic fields showed no parasites. Malaria parasites and leukocytes were counted in the same fields until 300 leukocytes were counted. Parasite densities were estimated by using an assumed count of 8,000 leukocytes/ $\mu$ L blood.

Blood samples were collected into EDTA tubes. At delivery, blood was collected from mothers of enrolled infants to assess viral load, whether syphilis was present, and hemoglobin level. One month postpartum, maternal venous blood was collected to determine counts of CD4- and CD8-positive T-lymphocytes (CD4+ and CD8+). Capillary blood was collected from infants by a heel prick

on the day of delivery and then monthly thereafter for HIV testing. Plasma was separated from blood samples and stored at  $-70^{\circ}\text{C}$ .

Infant HIV testing was done by polymerase chain reaction (PCR) of proviral DNA extracted from peripheral blood mononuclear cells (27). HIV testing of pregnant women used two rapid test methods: an initial SeroStrip HIV-1/2 (Saliva Diagnostic Systems Pte Ltd, Singapore) and a confirmatory Capillus HIV-1/HIV-2 (Cambridge Diagnostics, Wicklow, Ireland) on all SeroStrip-positive samples. Sequential testing of samples using both methods has a high sensitivity and specificity (Richard W. Steketee, pers. comm.). Western blot was performed on discordant samples.

Maternal CD4+ and CD8+ counts were assessed by using commercial, dual-label monoclonal antibodies (Becton-Dickinson Immunocytometry, San Jose, CA) and standard fluorescent-activated cell sorting (FACScan, Becton-Dickinson) analysis following whole-blood lysis (28). Maternal HIV-1 viral load was determined by the Roche Amplicor HIV-1 monitor test version 1.0 (Roche Diagnostics, Indianapolis, IN), with a quantification limit of 400 viral copies per milliliter.

Syphilis antibodies were detected by venereal disease research laboratory (VDRL) slide test (EUROTEX-VDRL, Euromedi equip Ltd., West Harrow, UK). Hemoglobin was measured to the nearest 0.1 g/dL using a Hemocue machine (Mission Viejo, CA).

### Definitions

An uncomplicated pregnancy was defined as a pregnancy without the presence of AIDS-defining symptoms, hypertension, preeclampsia, polyhydramnios, abnormal fetal presentation, history of a cesarean section, hemorrhage, or repeated spontaneous abortions ( $>2$ ). Placental parasitemia was defined as any plasmodial asexual form detected on a thick film. Maternal HIV infection was defined as a positive result on both rapid tests; women not reactive with the initial SeroStrip HIV-1/2 test were considered HIV uninfected. Women whose serostatus could not be determined (i.e., those with discordant results on the two rapid tests and an indeterminate status with Western blot) were excluded from analysis. Newborns were classified as normal birth weight if they weighed  $\geq 2,500$  g, regardless of gestational age, and low birth weight if they weighed  $< 2,500$  g. Preterm delivery was defined as occurring at  $< 37$  weeks of gestation. Small for gestational age was defined as sex-specific birth weight  $\leq 10$ th percentile for weight-for-gestational-age (29).

### Determining Infant HIV Status

An algorithm was developed to describe the perinatal HIV infection status of infants. Infants were classified as

having acquired HIV infection perinatally (e.g., in utero or during labor and delivery) if they met the following conditions: 1) died and by the time of death had  $\geq 2$  consecutive positive HIV PCR tests, the first of which was at  $\leq 4$  months of age; 2) were lost to follow-up but had had  $\geq 3$  consecutive positive PCR results, the first of which was at  $\leq 4$  months of age; 3) remained alive with continued and consistent positive PCR results, the first of which was at  $\leq 4$  months of age. Infants were considered negative for perinatally acquired HIV if they had  $\geq 3$  PCR tests performed on them, and all tests were negative, with at least one of the negative tests at  $\geq 4$  months of age. Because infants had to be  $\geq 4$  months old to be classified as uninfected, those who died or were lost to follow-up before 4 months of age were excluded. Infants who acquired HIV at  $\geq 5$  months of age were considered to have acquired HIV postnatally and were included as nontransmitters from the perinatal perspective. Infants for whom we had insufficient PCR data to determine their status were classified as indeterminate and were excluded. Mothers of infected infants were classified as transmitters and those of uninfected infants as nontransmitters.

## Data Analysis and Statistical Methods

### Univariate Analysis

Plasma virus levels load below the limit of quantification (400 copies/ $\mu\text{L}$ ) were assigned a value of 200 copies/ $\mu\text{L}$ ; plasma viral load results were then  $\log_{10}$ -transformed. We defined high-density placental parasitemia as  $\geq 10,000$  parasites/ $\mu\text{L}$ , which corresponded approximately to the uppermost quintile of parasite density. Univariate analyses were performed by using  $\chi^2$  or Fisher exact tests (for cross-tabulations with an expected value in any cell  $\leq 5$ ) to compare proportions for categorical variables;  $t$  tests were used to compare normally distributed continuous variables. Relative risks (RRs) were computed with their 95% confidence interval (CI) to measure the strength of the associations between potential risk factors and perinatal MTCT.

### Multivariate Analysis

To evaluate the effect of placental malaria on perinatal MTCT, a Poisson log-linear model containing placental malaria and maternal viral load as primary predictor variables was constructed by using backward elimination; adjusted RRs were computed. An interaction term between placental malaria density and maternal viral load was significant ( $p = 0.02$ ) and was retained in the model. Because women with and without placental malaria may have different risk factors for perinatal MTCT, we fit three separate multivariate models: all study women, women without placental malaria, and women with placental malaria. All tests

were two-sided; *p* values <0.05 were considered significant. Analysis was done using STATA (StataCorp. 2001. Stata Statistical Software: Release 7.0 College Station, TX) and SAS (Version 8.0, SAS Institute, Cary, NC).

## Results

### Study Population

A total of 829 mother-infant pairs were enrolled; 317 (38.2%) infants with incomplete follow-up or indeterminate HIV status were excluded, leaving 512 mother-infant pairs. Included and excluded women did not differ in age, level of education, mean maternal viral load, mean CD4+ counts, malaria rates, high-density malaria rates, proportion of low birth order (i.e., gravida  $\leq 2$  versus  $\geq 3$ ), and rates of episiotomy or perineal tear. However, excluded women were more likely to have infants born with low birth weight (9.1% versus 5.5%, *p* = 0.04). The baseline characteristics of the 512 women included in the analysis are shown in Table 1. Among these 512 women, 128 (25.0%) had placental malaria and 353 (84.4%) had anemia (hemoglobin <11 g/dL).

### Correlates of Perinatal MTCT

Of 512 mothers, 102 (19.9%) transmitted HIV to their infants perinatally. High maternal viral load ( $\geq 10,000$  viral copies/ $\mu$ L) and low CD4+ count (<200 cells/ $\mu$ L) were significantly associated with perinatal MTCT (Table 2). When analyzed as continuous variables, transmitting mothers had higher geometric mean viral loads than non-transmitting mothers (7,083 versus 1,378 copies/ $\mu$ L; *p*<0.001) and low mean CD4+ count (511 versus 657 cells/ $\mu$ L; *p*<0.001). Other characteristics significantly associated with perinatal MTCT included episiotomy or perineal tear, low birth weight or small-for-gestational-age infants, and being of low birth order (primigravid or secundigravid).

Maternal peripheral parasitemia at delivery was not associated with MTCT; however, placental malaria was associated with a 40% reduction in the risk for perinatal MTCT (RR 0.6, 95% CI 0.4 to 1.0, *p* = 0.05) (Table 2). Compared to women without placental malaria (perinatal MTCT rate = 21.9%), women with placental parasitemia at lower densities (<10,000 parasites/ $\mu$ L) had a lower rate of perinatal MTCT (11.5%); this was not true for women with

Table 1. Characteristics of HIV-positive women and their newborns participating in perinatal HIV transmission study, Kisumu, western Kenya, 1996–2001<sup>a</sup>

Characteristic	All women (N = 512) <sup>b</sup>
<b>Maternal sociodemographic</b>	
Luo ethnicity	86.5%
Mean age (y) $\pm$ SD	22.4 $\pm$ 4.4 (range 14–39)
Mean gravidity $\pm$ SD	2.3 $\pm$ 1.4 (range 1–9)
Primigravid	35.9%
Completed primary education ( $\geq 8$ y)	68.0% (n = 510)
No salaried employment	74.3%
Married	78.4%
<b>History of fever and treatment for malaria</b>	
History of fever previous week at screening	23.2% (n = 509)
History of fever a fortnight before delivery	28.0% (n = 511)
Treated with antimalarials in current pregnancy	30.9%
Treated with chloroquine during current pregnancy	16.6%
Axillary temperature $\geq 37.5^\circ\text{C}$ at screening	2.9% (n = 455)
<b>Laboratory</b>	
VDRL-positive	7.3% (n = 385)
Hemoglobin <11 g/dL at screening	84.4% (n = 418)
Hemoglobin <8 g/dL at screening	20.6% (n = 418)
Mean maternal CD4+ count (% <200 cells/ $\mu$ L) 1 mo postpartum	629 $\pm$ 334 (4.7%) (n = 464)
Mean maternal log <sub>10</sub> viral load at delivery (% below detection limit of 400 copies)	3.28 $\pm$ 0.92 (33.0%) (n = 455)
Peripheral parasitemia at screening	21.9% (n = 415)
Peripheral parasitemia at delivery	19.7% (n = 497)
Placental malaria	25.0%
<b>Delivery</b>	
Episiotomy or perineal tear	36.4%
Mean duration of rupture of membranes $\pm$ SD (% >4 hours)	2.7 $\pm$ 6.2 (15.4%)
<b>Newborn</b>	
Mean birth weight (% low birth weight)	3144 $\pm$ 420 (5.5%)
Prematurity (<37 wks completed gestation)	8.2%
Maternal HIV transmitters	102 (19.9%)

<sup>a</sup>VDRL, venereal disease research laboratory slide test.

<sup>b</sup>If characteristic not measured for all 512 women, n is given in parentheses.



Table 2. Risk factors associated with perinatal HIV infection by maternal viral, immunologic, obstetric, and other factors (univariate analysis), western Kenya, 1996–2001

Variable	No. studied	No. infected (%)	Relative risk (95% confidence interval)	p
Viral load >10,000				
No	358	50 (14.0)		
Yes	97	40 (41.2)	3.0 (2.1 to 4.2)	<0.001
CD4+ cells <200				
No	442	74 (16.7)		
Yes	22	13 (59.1)	3.5 (2.4 to 5.3)	<0.001
Hemoglobin <8 g/dL at screening				
No	332	58 (17.5)		
Yes	86	22 (25.6)	1.5 (1.0 to 2.3)	0.09
3rd-trimester maternal parasitemia				
No	324	64 (19.8)		
Yes	91	15 (16.5)	0.8 (0.5 to 1.4)	0.48
Maternal parasitemia at delivery				
No	399	83 (20.8)		
Yes	98	15 (15.3)	0.7 (0.4 to 1.2)	0.22
Placental malaria				
No	384	84 (21.9)		
Yes	128	18 (14.1)	0.6 (0.4 to 1.0)	0.05
Ever been treated for tuberculosis				
No	496	98 (19.8)		
Yes	13	4 (30.8)	1.6 (0.7 to 3.6)	0.33
Treated with chloroquine during pregnancy				
No	427	88 (20.6)		
Yes	85	14 (16.5)	0.8 (0.5 to 1.3)	0.38
Treated for vaginal discharge				
No	477	93 (19.5)		
Yes	32	9 (28.1)	1.7 (0.8 to 2.6)	0.24
Hospitalized during current pregnancy				
No	472	95 (20.1)		
Yes	39	7 (18.0)	0.9 (0.4 to 1.8)	0.74
History of fever 2 wks before delivery				
No	368	70 (19.0)		
Yes	143	32 (22.4)	1.2 (0.8 to 1.7)	0.39
Episiotomy or perineal tear				
No	325	56 (17.2)		
Yes	186	46 (24.7)	1.4 (1.0 to 2.0)	0.04
Primi- or secundigravid				
No	190	26 (13.7)		
Yes	322	76 (23.6)	1.7 (1.1 to 2.5)	0.007
Low birth weight				
No	484	91 (18.8)		
Yes	28	11 (39.3)	2.1 (1.3 to 3.4)	0.008
Prematurity				
No	468	92 (19.7)		
Yes	42	10 (23.8)	1.2 (0.7 to 2.1)	0.52
Small for gestational age				
No	444	83 (18.7)		
Yes	66	19 (28.8)	1.5 (1.0 to 2.4)	0.06

higher-density placental parasitemia ( $\geq 10,000$  parasites/ $\mu\text{L}$ , perinatal MTCT rate = 25.0%), who represented approximately one fifth of women with placental malaria. Among women with viral load below the detection level, perinatal MTCT occurred in none of 35 mothers with malaria, compared to 10 (8.7%) of 115 mothers without malaria ( $p = 0.12$ ).

#### Multivariate Analysis of Risk Factors for Perinatal MTCT

Among all study women, high maternal viral load and episiotomy or perineal tear were independent risk factors, and low-density (but not high-density) malaria was an independent protective factor for perinatal MTCT. Among women without microscopically detectable placental

Table 3. Multivariate analysis of risk factors for perinatal HIV transmission, western Kenya, 1996–2001

	Adjusted relative risks (ARR) for perinatal HIV transmission <sup>a</sup>					
	All women, <sup>b</sup> N = 454		Placental malaria–negative, n = 348		Placental malaria–positive, <sup>b</sup> n = 107	
	ARR (95% CI)	p	ARR (95% CI)	p	ARR (95% CI)	p
Log <sub>10</sub> viral load	1.8 (1.6 to 2.1)	<0.001	1.7 (1.4 to 2.0)	<0.001	3.5 (2.5 to 4.8)	<0.001
Episiotomy or perineal tear	1.6 (1.2 to 2.1)	0.004	–	–	4.8 (2.3 to 9.7)	<0.001
Low birth weight	–	–	1.9 (1.1 to 3.2)	0.03	–	–
Gravidity <3 versus ≥3	–	–	1.8 (1.2 to 2.8)	0.003	–	–
Placental malaria status						
Negative	Reference <sup>c</sup>	–	N/A	–	–	–
<10,000 parasites/μL	0.4 (0.2 to 0.6) <sup>b</sup>	<0.001	N/A	–	Reference	–
≥10,000 parasites/μL	0.7 (0.3 to 21.5) <sup>b</sup>	NS	N/A	–	2.0 (1.1 to 3.9)	0.04

<sup>a</sup>–, factor was not retained in the final model; CI, confidence interval; N/A, not applicable; NS, not significant.

<sup>b</sup>A significant interaction was found between viral load and placental malaria density ( $p = 0.02$ ) in these analyses. The effect of this interaction on the relative risk for placental malaria is shown in Figures 1 and 2. All relative risks given in this table do not include this interaction but give a weighted average of the placental malaria effect at various levels of viral load.

<sup>c</sup>An alternative model, in which placental malaria was fit as a binary variable (positive or negative), showed that placental malaria was protective for perinatal HIV transmission (relative risk 0.4, 95% CI 0.3 to 0.7,  $p < 0.001$ ). In that model, log<sub>10</sub> viral load and episiotomy or perineal tear remain independent risk factors.

malaria, high maternal viral load, gravidity  $\leq 2$ , and low birth weight in the newborn were significant risk factors or markers for perinatal MTCT. Among women with placental malaria (where perinatal MTCT was overall lower than in the malaria-negative women), high maternal viral load, episiotomy or perineal tear, and high-density placental infection were significant risk factors for perinatal MTCT (Table 3). In both the multivariate models for the entire population and women with placental malaria, significant interaction was found between viral load and placental malaria density.

In further examination of the interaction between maternal viral load and placental malaria, we found no significant differences in the frequency of episiotomy or perineal tear, mean CD4+ count, mean maternal hemoglobin level, mean birth weight, and frequency of small-for-gestational-age neonates among women without malaria, those with low-density placental malaria, and those with high-density placental malaria. Women with placental malaria were of lower mean gravidity than women without malaria (2.1 vs. 2.4,  $p = 0.03$ ). Geometric mean maternal viral load was slightly higher in women with low-density placental malaria (2,226 copies/μL) and was nearly twofold higher in women with high-density placental malaria (3,390 copies/μL) than the viral load in women without placental malaria (1,774 copies/μL); however, these differences were not significant ( $p = 0.14$ ). Viral load

was significantly higher among women with peripheral malaria parasitemia at delivery (2,979 copies/μL) than among women without (1,725 copies/μL,  $p = 0.03$ ). As shown in Table 4, the geometric mean viral load was five-fold higher in transmitters than in nontransmitters. While in women without placental malaria the geometric mean viral load in transmitters was fourfold higher than in nontransmitters, among women with malaria, the geometric mean viral load was 25-fold higher in transmitters than in nontransmitters. Among transmitting women, those with placental malaria had an eightfold higher geometric mean viral load than those women without placental malaria.

These associations were further evaluated in models comparing the relative risk for perinatal transmission between the three groups with placental malaria at various levels of maternal viral load. Low-density placental parasitemia was associated with significant protection for perinatal MTCT at the lower viral load levels, but not at higher viral load levels (Figure 1). When women with low- and high-density parasitemia were compared (Figure 2), high-density placental parasitemia was associated with increased risk for perinatal MTCT but only at the higher viral load levels.

## Discussion

This evaluation of perinatal HIV transmission in a malarious area of western Kenya demonstrated that

Table 4. Association between maternal viral load and placental malaria among women who did and did not transmit HIV perinatally to their infants, western Kenya, 1996–2001

	Geometric mean HIV viral load <sup>a</sup>		p value <sup>b</sup>
	Transmitters	Nontransmitters	
All women (N = 455)	7,083	1,378	<0.001
Placental malaria–positive	41,217	1,675	<0.001
Placental malaria–negative	5,402	1,286	<0.001
p value	0.002	0.26	

<sup>a</sup>Viral load expressed as copies per microliter of plasma.

<sup>b</sup>p from *t* test.

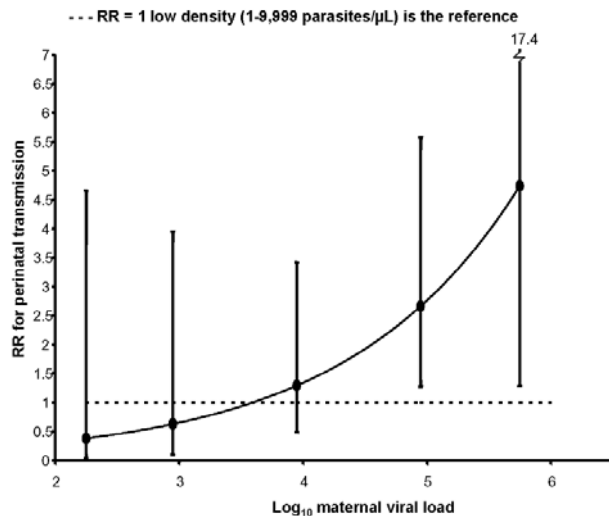


Figure 2. The effect of viral load and high-density placental malaria on risk for perinatal HIV transmission, western Kenya, 1996–2001. Women with high-density placental malaria ( $\geq 10,000$  parasites/ $\mu\text{L}$ ) are compared to those with low-density placental malaria ( $< 10,000$  parasites/ $\mu\text{L}$ , represented by the horizontal dashed line). RR, relative risk. Error bars refer to 95% confidence interval.

approximately 20% of infants born to HIV-infected mothers acquired HIV by 4 months of age, similar to rates reported in other sub-Saharan African settings (30–32). Consistent with other studies, we found that maternal viral load in peripheral blood at the time of delivery and having an episiotomy or perineal tear were risk factors for perinatal MTCT (33,34). Contrary to our expectations, we observed that women with placental malaria had lower rates of perinatal MTCT than women without placental malaria. However, women with high-density malaria had significantly higher rates of perinatal MTCT than parasitemic women with low-density malaria. In additional models, only low placental parasite density (1–9,999 parasites/ $\mu\text{L}$  of blood) was associated with protection from perinatal MTCT; high-density placental infection was either a risk factor for perinatal MTCT (in the model evaluating only women with malaria) or it was a neutral factor (in the model evaluating all women). Maternal viral load was slightly higher in women with high-density placental malaria, and, among transmitting women, mean viral load was eightfold higher if the woman had concurrent placental malaria, consistent with the suggestion that high-density malaria may be an important stimulus of viral replication (10–12).

Our study had some important limitations. First, only healthy women were screened for this study; no women with AIDS or any known underlying chronic illness were enrolled. Although this eliminated potential conditions that could have complicated the analysis (e.g., a higher likeli-

hood of additional concurrent infections), it restricted our study population to the “healthiest” women with HIV and likely resulted in an underestimate of the overall rate of perinatal MTCT in our study area. Some infants categorized as having acquired HIV through perinatal transmission may have actually acquired it through early breastfeeding transmission. However, including these infants would likely bias the results toward underestimating the magnitude of the observed risks. Our measurement of placental malaria was limited to microscopy examination of placental smears and could have been inexact; those with no evidence of malaria may have had very low-density infection and may have been misclassified as having no malaria. Such misclassification would be expected to bias our findings toward the null hypothesis. Because placental malaria can cause an inflammatory response in the placenta, the use of leukocyte count to calculate parasite density may have resulted in an underestimation. However, our cutoff of 10,000 parasites/ $\mu\text{L}$  for high-density placental parasitemia is essentially a relative measure based on the upper quintile of densities; therefore, we do not think that our estimation technique would have introduced any bias. Finally, in a study such as ours, loss to follow-up always has the potential to introduce bias. Approximately one third of infants enrolled in our study were lost to follow-up. However, as noted, these mothers and infants were generally similar to the study population included in our analysis, and we were unable to detect biases that would have affected our analysis.

Our observation of an association between low-density placental malaria and reduced perinatal MTCT has several

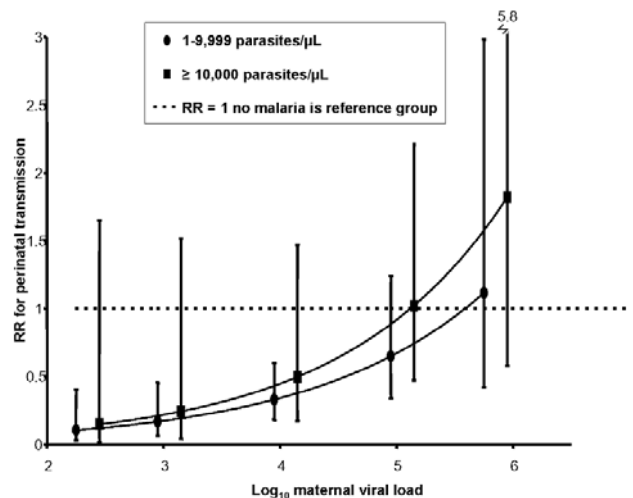


Figure 1. The effect of viral load and placental malaria density on risk for perinatal HIV transmission, western Kenya, 1996–2001. Women with low- ( $< 10,000$  parasites/ $\mu\text{L}$ , circles) and high- ( $\geq 10,000$  parasites/ $\mu\text{L}$ , squares) density placental malaria are compared with women without placental malaria (represented by the horizontal dashed line). RR, relative risk. Error bars refer to 95% confidence interval.

possible explanations. First, over 16% of women reported self-treatment (typically for fever) with chloroquine during pregnancy. High-grade chloroquine resistance in this area is widespread; its use is unlikely to clear placental infections, but it may reduce parasite densities. Chloroquine is known to have anti-HIV properties and to reduce HIV-1 replication and viral loads in adults (35,36) and as such could potentially reduce the risk for perinatal MTCT. Although we identified no differences in the proportion of women with or without placental malaria who used chloroquine, and no association was found between chloroquine usage and MTCT, women identified as having placental malaria may have used chloroquine more frequently and at higher doses (information that was not collected) than women without placental malaria. A second possibility is that some of the women classified as having placentas negative for malaria parasites may actually have had low-level chronic malaria associated with inflammation and increased HIV replication. In contrast, women classified as having low-density malaria may have had recent malaria with minimal inflammatory response and thus no increase in HIV replication. Unfortunately, blood films are not capable of detecting inflammatory responses.

A more likely explanation is that a balance exists in the uterine-placental-fetal environment among malaria-induced antigen stimulation, HIV viral replication, maternal host immune response to both malaria and HIV, and the likelihood of MTCT. Although high viral load has been shown to increase the risk for MTCT, no more than half of exposed infants, even at high maternal viral load, become infected with HIV-1. These data suggest that other systemic or placental factors must be important in preventing HIV-1 transmission. Recent studies suggest that selected cytokines and hormones potentially affect HIV-1 transplacental transmission and that both innate and acquired protection play a role in MTCT (37). First, malaria is known to induce disequilibrium in the balance between Th1 and Th2 responses, favoring the Th1 pathway (38–40). T-helper responses are known to control HIV replication; hence, inducing Th1 response in the placental compartment could lead to reduced HIV-1 replication (41,42). Indeed, a moderate increase was found in the Th1 cytokine interferon- $\gamma$  response in the intervillous blood mononuclear cell responses of HIV-positive mothers with placental malaria as compared to HIV-positive mothers without placental malaria (43). Second, leukemia inhibitory factor induces a potent inhibition of HIV replication, and this cytokine is upregulated in placentas of women who do not transmit HIV (44). Malarial antigens may induce production of leukemia inhibitory factor that results in reduced rates of perinatal MTCT. Third, malarial antigen may result in altered chemokine production, which in turn can block chemokine receptors necessary for cellular HIV entry (45).

Recent immunologic studies conducted in a group of women who participated in this cohort showed that macrophage inflammatory protein (MIP)-1 $\beta$ , a chemokine known to block the entry of HIV-1, was significantly elevated in the intervillous blood plasma of women with placental malaria irrespective of their HIV status. In women with concurrent HIV and placental malaria, the intervillous blood plasma levels of MIP-1 $\beta$  were significantly elevated compared to HIV-negative women with placental malaria and HIV-positive women without placental malaria (46). Thus, a complex balance of antigen stimulus and immune response may occur in the placenta, as demonstrated in recent studies focusing on the placental intervillous blood responses (38–40,43,46). In this scenario, active placental malaria might lead to one of the local immune responses described. This response may control the density of parasitemia and may also provide immune control that limits perinatal MTCT. For most women with placental malaria (approximately 80% in our study), this resultant balance leads to “protection” with controlled (low-density) malaria and reduced perinatal MTCT. For a minority of women with malaria (approximately 20% in our study), the balance is tilted to inadequate control of placental malaria (i.e., higher density of malaria parasites with concomitant increased antigen stimulation of viral replication and higher viral load) and higher rates of perinatal MTCT. This finding is consistent with our observation that women with high-density placental malaria do have higher rates of perinatal MTCT than women with low-density placental malaria, but that they require much higher viral loads to achieve this transmission. Our findings suggest complex relationships between malaria, viral load, and perinatal MTCT. These findings will need to be confirmed and expanded upon in further research in different settings.

The finding of reduced perinatal MTCT in the presence of low-density, but not high-density, placental malaria does not suggest altering existing recommendations for the use of intermittent preventive antimalarial treatment in pregnant women in malarious areas of Africa (47). For most dually (malaria and HIV) infected women, the overall outcome would have modest benefit in reducing perinatal MTCT, but for the minority with insufficiently controlled infections, perinatal MTCT would be increased. At this point, we cannot differentiate in advance which women will end up in which group. The important benefit of anti-malarial treatment may be to reduce the likelihood of women having high-density placental malaria and in also helping reduce the other known adverse effects of malaria during pregnancy, including anemia, low birth weight, and prematurity (1).

As noted earlier, this study was carried out in Kenya in a changing environment of perinatal HIV and malaria prevention and has been transformed into a program deliver-



ing short-course antenatal antiretroviral therapy to HIV-infected women and a system to support providing intermittent preventive antimalarial treatment during pregnancy according to the newly adopted national policy (48). As more countries adopt policies of antiretroviral and antimalarial interventions in pregnancy, the interrelationship and ultimate benefit of these two interventions need to continue to be evaluated.

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# Ixodid and Argasid Tick Species and West Nile Virus

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Control of West Nile virus (WNV) can only be effective if the vectors and reservoirs of the virus are identified and controlled. Although mosquitoes are the primary vectors, WNV has repeatedly been isolated from ticks. Therefore, tick-borne transmission studies were performed with an ixodid (*Ixodes ricinus*) and an argasid tick species (*Ornithodoros moubata*). Both species became infected after feeding upon viremic hosts, but *I. ricinus* ticks were unable to maintain the virus. In contrast, *O. moubata* ticks were infected for at least 132 days, and the infection was maintained through molting and a second bloodmeal. Infected *O. moubata* ticks transmitted the virus to rodent hosts, albeit at a low level. Moreover, the virus was nonsystemically transmitted between infected and uninfected *O. moubata* ticks co-fed upon uninfected hosts. Although ticks are unlikely to play a major role in WNV transmission, our findings suggest that some species have the potential to act as reservoirs for the virus.

The first report of a West Nile virus (WNV) outbreak within the Western Hemisphere occurred in 1999 in New York City and resulted in human, equine, and avian deaths (1). Since 1999, WNV has been found in an additional 44 states of the United States as well as in parts of Canada, the Caribbean, and South America (2,3). During 2002 more than 4,000 people diagnosed with WNV and 284 deaths were reported in the United States (latest records available from: <http://www.cdc.gov/ncidod/dvbid/westnile/index.htm>).

WNV is a member of the genus *Flavivirus* that contains over 70 identified viruses. Most of these viruses are vectored by mosquitoes or ticks, although a few have no known vectors (4). WNV has been isolated from 43 species of mosquito in the United States (5), the most important of which is *Culex pipiens* (6). It has also been isolated from hard (ixodid) and soft (argasid) tick species in regions of Europe, Africa, and Asia (7–13) where WNV is endemic. Ticks rank second only to mosquitoes in their importance as vectors of human pathogens and transmit a greater variety of infectious agents than any other arthro-

pod group (14). However, whether or not ticks are major vectors of WNV has not been adequately investigated.

Current strategies to control WNV in the United States are largely based on measures to avoid exposure and to control vector species, but at present only mosquito species are targeted by government surveillance and preventive control programs (15). Resident U.S. tick populations could also play a role in the WNV transmission cycle in the current outbreak. We investigated an argasid tick species and an ixodid tick species for their competence as vectors and reservoirs of the New York strain (NY99) of WNV.

## Materials and Methods

### Ticks

We tested a hard tick species, *Ixodes ricinus*, and a soft tick species, *Ornithodoros moubata*, for their vector competence with WNV (NY99 strain). These species are not native to the United States and were chosen mainly for their availability. *O. moubata* ticks were considered potential vectors for the Eg101 strain of WNV in a study by Whitman and Aitken in 1960 (16). *I. ricinus* ticks are the primary vectors of *Borrelia burgdorferi*, the agent causing Lyme disease in Europe and are important vectors of the flaviviruses tick-borne encephalitis virus (TBEV) and Louping ill virus (LIV) (17).

Ticks were taken from colonies reared and maintained for many generations at the Centre for Ecology and Hydrology, Oxford, according to standard methods (18). Colony ticks were WNV negative by reverse transcriptase–polymerase chain reaction (RT-PCR) testing (15 members of each species tested).

### Virus and Viral Assays

The WNV strain used (NY99) was supplied by Robert Shope, University of Texas. High-titer mouse brain suspension stocks of WNV ( $2.9 \times 10^7$  PFU/mL<sup>-1</sup>) were diluted in phosphate-buffered saline (PBS) to a concentration of  $10^5$  PFU/mL<sup>-1</sup> before use. Viral stocks and the serum samples from infected mice were tested for infectious virus

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by plaque assays on pig kidney epithelial cells as described previously (19), by using a 3% carboxymethylcellulose overlay.

### Tick Infection and Co-feeding Transmission Experiments

Seven groups of six BALB/c mice (female, 4–6 weeks old) were injected subcutaneously with  $10^4$  PFU of WNV. Three of the mice were bled daily from the tail to follow the course of viremia by plaque assay. Two groups of mice were infested with *I. ricinus* nymphs (20 per mouse); one group was infested 3 days before infection, the other 4 days after infection. The other five groups of mice were infested with second instar *O. moubata* ticks (10 per mouse) on either the same day (day 0) or 1, 2, 3, or 4 days after infection. After the initial experiment, and to increase the number of positive ticks available for experimentation, 12 additional mice were infested with *O. moubata* 2 days after infection with WNV.

Ticks housed in gauze-covered neoprene feeding chambers on mice (18) were removed when fully engorged, 24 hours after infestation in the case of *O. moubata* ticks and 6 days after infestation in the case of *I. ricinus* nymphs. The engorged ticks were stored at 20°C in KCl-saturated desiccators until testing for WNV or until ready for a further bloodmeal, as indicated in Table 1. After storage, the ticks (pools and individual ticks) were homogenized in 500  $\mu$ L of PBS by using plastic homogenizers under sterile

conditions. The homogenates were frozen and stored at  $-70^\circ\text{C}$  until analyzed. Tick homogenates were assayed for infectious virus antigen (by immunofluorescence assay) and viral RNA (by RT-PCR) as shown in Table 1.

Co-feeding transmission experiments were carried out by infesting clean BALB/c mice ( $n = 7$ , Harlan, UK) with 10 third instar *O. moubata* ticks 57 days after they had taken an infectious bloodmeal, and 10 uninfected ticks (second instar) in separate feeding chambers. The two feeding chambers were separated by at least 1 cm.

To investigate tick-to-host transmission, 10 BALB/c mice were infested with cohorts of 5, 10, 15, or 20 third instar *O. moubata* ticks 57 days after an infectious bloodmeal. Fifteen days after infestation, the mice (including those used for co-feeding) were euthanized; brains were removed, homogenized in 1 mL of sterile PBS, and stored at  $-70^\circ\text{C}$  until they were tested for WNV.

### Immunofluorescence Assay

Samples of tick (or mouse brain) homogenate (100  $\mu$ L) were used to infect  $2 \times 10^6$  C6/36 mosquito cells in a total of 3 mL L-15 medium containing 7% fetal calf serum (Gibco-BRL, Paisley, UK) in 30 mm Petri dishes that contained glass coverslips. Infected cells were incubated at 28°C for 3 days. Cells were fixed in cold acetone and treated according to standard methods (19). Infected cells were fluorescein isothiocyanate-labelled with a broadly reactive flavivirus E-protein monoclonal antibody (MAb 813) (20)

Table 1. Results of immunofluorescence assay (IFA) or nested reverse transcriptase-polymerase chain reaction (RT-PCR) from *Ornithodoros moubata* and *Ixodes ricinus* ticks fed on West Nile virus-inoculated BALB/c mice or noninfected mice (co-fed ticks)

Species	Developmental stage	Days from infection		IFA <sup>b</sup> +/-	RT-PCR <sup>c</sup> (no. positive/no. tested)	
		to infestation	Days after engorgement <sup>a</sup>			
<i>O. moubata</i>		0	1, 2, 7	– (8)	ND	
First bloodmeal (infected mice)	Second instar	1	1–7	– (5)	ND	
		2	1–7, 14	+ (5)	+ (5)	
		3	1–7, 14	+ (5)	+ (5)	
		4	1, 3, 7	– (5)	ND	
	Third instar	2	22	+ (5)	ND	
		3	22	+ (5)	ND	
		2	132	+ (5)	7/14	
Second bloodmeal (uninfected mice)	Third instar	2	60 (3)	+ (5)	+ (5)	
		2	64 (7)	+ (5)	+ (5)	
		2	75 (25)	+ (5)	+ (5)	
Uninfected co-fed <i>O. moubata</i> ticks	Second instar	N/A	5	ND	15/66	
	Third instar	N/A	45	ND	4/15	
<i>I. ricinus</i>	Nymph	4	2	ND	0/12	
		First bloodmeal (infected mice)	–3	2	ND	2/12
		–3	30	ND	0/25	
BALB/c mice <sup>d</sup>	N/A	N/A	N/A	– (1)	1/17	

<sup>a</sup>Number of days after the ticks had completed feeding on inoculated mice when ticks were tested for virus infection. Where given, parentheses depict ticks that had fed a second time and the number of days after which the ticks were tested.

<sup>b</sup>Tick homogenate samples were scored positive if >10% of inoculated C6/36 cells showed specific fluorescence with both 813 and 546 monoclonal antibodies. Numbers of ticks in each pool are shown in parentheses.

<sup>c</sup>Where indicated by +, pools of ticks were tested; numbers of ticks in each pool are shown in parentheses. ND, not done.

<sup>d</sup>Mice were infested with infected *O. moubata* ticks and after 14 days were sacrificed and the brain homogenates tested by IFA and RT-PCR. N/A, not applicable.



Table 2. Nucleotide sequences of primers used in first round (5'[1] and 3'[2]) and second round (5'[2] and 3'[2]) of nested RT-PCR<sup>a</sup>

Primer	Sequence	Position in WNV (NY99) <sup>b</sup>
5'(1)	CCATATGAATTCCATGAGTGCTATCAATCGGCGGAG	31 aa upstream (C gene) from start of PrM gene (373)
3'(1)	CATATGCGGCCGCTTACTAGGTGATTGATCTGTTGTTCTCC	31 aa downstream (NS1) from end of E gene (2,562)
5'(2)	CATATGCGGCCGCTTACTACCGGTCCATCCAAGCCTC	Start of E gene (967)
3'(2)	CCATATAGATCTCGGAGGTCATTCAACTGCCTTGAATGAGC	395 aa into E gene (2,152)

<sup>a</sup>RT-PCR, reverse transcriptase–polymerase chain reaction; aa, amino acids.

<sup>b</sup>Nucleotide position in relation to complete genome sequence of WNV (NY99) shown in parentheses (accession no. AF19685).

or a WNV-specific monoclonal antibody (MAb 546) (21). Labeled cells were visualized by using an Olympus epifluorescence microscope. Uninfected cells were used as negative controls and cells infected with the original viral stock as positive controls. Tick samples were deemed positive when more than 10% of the cells showed specific fluorescence with both monoclonal antibodies.

### Nested RT-PCR Assay

RNA was extracted from homogenized samples (100  $\mu$ L) by using RNeasy total RNA extraction kit in accordance with the manufacturer's instructions (Promega, Madison, WI). cDNA synthesis was carried out with Superscript II reverse transcriptase (Invitrogen, San Diego, CA) and 3'(1) primer (Table 2) for 50 min at 42°C, in a total volume of 20  $\mu$ L. PCR was carried out on the cDNA (1  $\mu$ L) by using 5'(1) and 3'(1) primers. Nested PCR was carried out on 1  $\mu$ L of the first-round PCR product using the nested primers 5'(2) and 3'(2). All PCR reactions were carried out in a 50- $\mu$ L volume with REDTaq DNA polymerase (Sigma Chemical Co., St. Louis, MO). A Hybaid Touchdown thermal cycler was used with the following program: 94.5°C for 1 min, 30 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. Viral stock, RNA extracted from uninfected ticks, and PBS-only samples were used as control reactions. Positive samples gave a PCR product of approximately 1.2 kbp. This method could detect RNA from a viral stock equivalent of 9 PFU (data not shown).

To confirm the identity of RT-PCR products, PCR products were gel purified with QIAquick (Qiagen, Crawley, UK) columns in accordance with manufacturer's instructions. The purified DNA was sequenced with an ABI automatic sequencer and the nested primers 5'(2) and 3'(1) and a primer based on the internal sequence of the E gene of WNV (not shown).

## Results

### Host-to-Tick Transmission

BALB/c mice infected with WNV were weakly viremic 2 and 3 days after injection, with mean titers of  $6 \times 10^3$  and  $3 \times 10^3$  PFU/mL<sup>-1</sup> blood respectively. After 4 days, viremia was no longer detectable by plaque assay, although severe

neurologic disease developed in the mice after 5 or 6 days, and they were euthanized. *O. moubata* ticks that had fed on mice on days corresponding to the viremic period (i.e., days 2 and 3 after infection), but not those fed outside this period, contained viral antigen as measured by immunofluorescence assay (IFA) (Table 1). Two days after engorgement, 17% (n = 12) *I. ricinus* ticks that started to feed on hosts 3 days before WNV injection, but not those that had started to feed 4 days after injection, were positive for WNV RNA. When the former group of ticks was tested 28 days later, no evidence of infection was found. Infected *O. moubata* ticks, in contrast, maintained the virus after molting into the next instar (i.e., third instar); following a second, noninfectious bloodmeal; and after molting a second time into fourth instars. Fifty percent of the individual ticks (n = 14) tested by RT-PCR were positive for WNV RNA when examined 132 days after the initial infectious bloodmeal.

### Co-feeding Transmission

Five days after engorgement, 23% (n = 66) of uninfected second instar *O. moubata* ticks that had co-fed with infected cohorts of third instar ticks on noninfected mice were positive for WNV RNA (Table 1). The remaining uninfected ticks (n = 15) were tested after they had molted into third instars, 45 days after co-feeding. Four of these ticks (26%) were positive for WNV RNA. The identities of the PCR products obtained from three positive samples were confirmed by sequence analysis.

### Tick-to-Host Transmission

Infected cohorts of *O. moubata* ticks (third instar) were fed on uninfected mice to investigate tick-to-host transmission. Of the 17 uninfected mice used (including mice used in co-feeding experiments), none showed clinical signs of infection. One of the brains tested, from a mouse infested with an infected cohort of 20 ticks, was positive by RT-PCR but negative when tested by IFA (Table 1). The PCR product was sequenced to confirm the identity of WNV.

## Discussion

Laboratory studies from the 1950s suggested that some tick species might serve as competent vectors for WNV. Hurlbut and Taylor (1956) showed that *O. savignyi* and *O. erraticus* ticks were infected after feeding on mice inocu-

lated with the Ar-248 strain of WNV, but transmission from infected ticks to mice was not observed (22,23). Vermeil et al. (1959) infected *O. maritimus* and *O. erraticus* ticks by feeding on inoculated (Uganda 28B strain) chickens, guinea-pigs, mice, or gerbils. Infected ticks transmitted the virus to uninfected mice (24). More recently, an artificial membrane system was used to infect *Argas arboreus* ticks, which were then able to transmit the virus to uninfected hosts, although transstadial transmission of WNV was not observed (25,26).

Our study demonstrated that both *I. ricinus* and *O. moubata* ticks become infected with WNV (NY99 strain) through feeding on virus-infected rodent hosts, but only when these hosts were viremic (i.e., systemic transmission). Thirty days after engorgement, we no longer found any evidence of WNV infection in the *I. ricinus* ticks. This finding suggests that nymphs of this tick species do not support replication of the virus, and therefore are not competent vectors for WNV. By extrapolation, the closely related tick species, *I. scapularis* (the main U.S. Lyme disease vector) is also unlikely to be a competent vector of WNV, although this hypothesis will need to be confirmed experimentally.

In contrast, infected *O. moubata* ticks maintained infectious virus for at least 132 days (length of experiment), and WNV persisted transstadially through at least two developmental stages. Evidence for tick-to-host transmission of WNV was found in our study, although the level of infection observed (subclinical) makes assessing its importance without further investigation difficult. Whitman and Aitken (1960) observed much higher levels of transmission from WNV-infected (Eg101 strain) *O. moubata* ticks to day-old chicks but only when very high feeding densities were used (an average of 49 ticks per chick) (16). Although ticks often feed in large numbers on individual hosts (27), tick-to-host transmission appears to be very inefficient when compared to mosquito transmission of WNV (23). Consequently, this mode of transmission is unlikely to be important in the natural transmission cycle of WNV. Perhaps higher levels of infection (and therefore transmission) would be found with ticks that feed on birds, the natural reservoir hosts of WNV. Some avian species exhibit much higher ( $>10^{10}$  PFU/mL serum) and more prolonged viremia when infected with WNV than the mice used for this investigation (28,29). Although neither of the tick species that we tested are obligate bird feeders, *I. ricinus* ticks often feed on pheasants in the United Kingdom (30), and several species of *Ornithodoros* ticks feed almost exclusively on birds, for example, the *O. capensis* group of ticks that are established along the southern coast of the United States (31). As members of this group have been shown to be competent vectors for WNV (24), these ticks could represent a reservoir of the virus in the United States.

The transmission of flaviviruses such as TBEV and LIV from infected to noninfected ixodid ticks through co-feeding on nonviremic hosts (nonsystemic transmission) is a well-established phenomenon (32). Indeed, this mode of transmission is believed to play a substantial role in the epidemiology of these diseases (27). We tested for co-feeding transmission of WNV between infected and uninfected *O. moubata* ticks. More than 22% of the uninfected ticks were positive for WNV RNA 5 days after co-feeding. A similar percentage of ticks were positive 40 days later, after having molted to the next developmental stage. As co-fed ticks were in contact with the mice for  $<24$  hours, this finding strongly suggests that WNV was nonsystemically transmitted between infected and uninfected ticks, since viremia had insufficient time to develop. Our study represents the first unequivocal report of co-feeding transmission by an argasid tick species. Argasid ticks, unlike ixodid ticks, typically feed for  $\leq 2$  hours. Vesicular stomatitis virus has been transmitted between infected and noninfected co-feeding black flies (*Simulium vittatum*), insects that typically feed for 4–5 min (33). Langerhans cells are believed to be the agents of viral transmission between feeding sites of infected and noninfected co-feeding hard ticks (32,34). Langerhans cells, which are susceptible to WNV infection (35), have been shown to migrate rapidly (within 2 hours) from localized antigen-stimulated epidermal sites (36). Therefore, these cells could possibly play a similar role in the co-feeding transmission of WNV by soft tick species.

Although this study is not exhaustive, it does demonstrate that tick species can become infected with the U.S. strain of WNV through feeding upon infected hosts and through co-feeding with infected ticks on noninfected hosts. In some tick species, WNV can be maintained through the transstadial stages of the tick lifecycle, and infected ticks may be capable of infecting hosts through further feeding. When compared to experimental studies with mosquito species (37–39), ticks are clearly not efficient vectors of WNV and therefore are unlikely to be important vectors for WNV in the current U.S. epidemic. However, our results demonstrate that WNV can persist for a comparatively long time in infected ticks and be transmitted between vertebrate hosts; this finding suggests a reservoir potential of ticks for WNV that justifies further investigation.

Dr. Lawrie is a postdoctoral researcher in the Nuffield Department of Clinical Laboratory Sciences, University of Oxford. His research interests include identifying and characterizing cancer-associated antigens that are recognized by autologous antibody responses, molecular aspects of the tick-host interface, and transmission of flaviviruses in tick species.

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# Antigenic and Genetic Variability of Human Metapneumoviruses

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Human metapneumovirus (HMPV) is a member of the subfamily *Pneumovirinae* within the family *Paramyxoviridae*. Other members of this subfamily, respiratory syncytial virus and avian pneumovirus, can be divided into subgroups on the basis of genetic or antigenic differences or both. For HMPV, the existence of different genetic lineages has been described on the basis of variation in a limited set of available sequences. We address the antigenic relationship between genetic lineages in virus neutralization assays. In addition, we analyzed the genetic diversity of HMPV by phylogenetic analysis of sequences obtained for part of the fusion protein (n = 84) and the complete attachment protein open reading frames (n = 35). On the basis of sequence diversity between attachment protein genes and the differences in virus neutralization titers, two HMPV serotypes were defined. Each serotype could be divided into two genetic lineages, but these did not reflect major antigenic differences.

Human metapneumovirus (HMPV) has recently been identified as a causative agent of respiratory tract illnesses in humans worldwide (1–3) and is a member of the *Pneumovirinae* subfamily within the *Paramyxoviridae* family (4). The *Pneumovirinae* subfamily consists of two genera: the pneumoviruses and the metapneumoviruses. Human respiratory syncytial virus (HRSV), the major viral cause of severe respiratory tract illnesses in children, is the type species of the pneumoviruses (5). Avian pneumovirus (APV), the causative agent of respiratory tract illnesses in turkeys and chickens (6), was the sole member of the *Metapneumovirus* genus until the discovery of HMPV (7).

For most pneumoviruses, different subgroups or subtypes have been identified. For HRSV, two subgroups have been identified on the basis of differences in nucleotide sequences, reactivity patterns with monoclonal antibodies, and in vitro neutralization assays with subgroup-specific

antisera (8–11). Additional genotypes have been identified within subgroups, largely on the basis of the high variability of the attachment protein gene (12,13). The fusion (F) and the attachment (G) proteins are the main targets for the neutralizing and protective antibody response (14–16), with F being one of the most conserved proteins and G the most variable (17–20). For APV, two different subgroups (A and B) have been defined on the basis of nucleotide sequences of the G protein and neutralization tests by using monoclonal antibodies that also recognize the G protein, but these subgroups belonged to one serotype (21). APV type C, a possible second serotype, was identified based on the lack of cross-reactivity with antisera specific for groups A and B, and the nucleotide sequences also proved to be substantially different from strains belonging to group A or B (22,23). In addition, subgroup D may exist, which contains isolates from France that are not neutralized by monoclonal antibodies raised against viruses belonging to either subgroup A, B, or C (24).

For HMPV, two major genetic lineages have been identified worldwide on the basis of analysis of a limited set of sequences (25–27). One feature of HMPV that poses a challenge in developing a future vaccine is that infections may occur in the presence of preexisting immunity. Very young children (<1 year) have been infected by the virus, and reinfections have also been demonstrated (28). HMPV might cause repeated infections throughout life, similar to HRSV, which could be either due to incomplete immunity or to genetic heterogeneity of the virus.

To develop vaccines, the extent of genetic and antigenic variability of the different HMPV transmembrane glycoproteins must be understood. We analyzed the genetic diversity of HMPV by phylogenetic analyses of sequences obtained for part of the F (n = 84) and the complete G open reading frames (ORFs) (n = 35). In addition, we addressed the antigenic relationship between the different lineages with virus neutralization assays using lineage-specific antisera raised in ferrets. Virologic studies have used a definition of a homologous-to-heterologous

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virus neutralization titer ratio of >16 as a definition for serotypes (29). On the basis of our results and the described definition, we now define the two major lineages of HMPV as serotype A and B. In accordance with the definition and our results, the sublineages within each serotype are not identified as different serotypes. At least two serotypes of HMPV are present in the human population, a finding that has implications for developing intervention strategies, such as immunization and vaccination.

## Materials and Methods

### Sample Collection, RNA Isolation, RT-PCR Assays, and Sequencing

HMPV-positive nasopharyngeal aspirate samples were obtained from different cohort studies: 61 samples from the Netherlands, 11 samples from Finland, 8 samples from England, 1 from Hong Kong, and 2 from Brazil. Clinical samples had been obtained from 1981 to 2002. Samples were obtained from young children, infants, adults, the elderly, and immunocompromised persons, who had mild to severe respiratory tract illnesses. Epidemiologic and clinical data for most isolates have been described elsewhere (30–32).

Similar to the influenza nomenclature, sequences are identified by country of origin, identification number, and year of isolation. RNA isolation was performed as described previously (25). cDNA was synthesized at 42°C for 60 min with random hexamer primers (Promega, Leiden, the Netherlands) and superscript II RNase H-reverse transcriptase (RT) (Invitrogen, Merelbeke, Belgium). An aliquot of cDNA was used in a polymerase chain reaction (PCR) assay to amplify the full-length G ORF or a fragment of the F ORF. Primers: SH7: 5'-TACAAAACAAGAACATGGGACAAG-3' and SH-8 5'-GAGATAGACATTAACAGTGGATT-3' (G ORF), BF100 5'-CAATGCAGGTATAACACCAGCAATATC-3', and BF101 5'-GCAACAATTGAACTGATCTTCAGGAAAC-3' (F ORF). Thermocycling was performed under the following conditions: 94°C for 1 min, 40°C for 2 min, 72°C for 3 min (40 cycles). When necessary, a nested PCR was performed by using 5 µL of PCR product with primers SH7 and SH8 for the G ORF or primers BF103 5'-ACATGCCAACATCTGCAGGACAAATAAAAC-3' and BF104 5'-ACATGCTGTTACCTTCAACTTTGC-3' for the F ORF. PCR products were sequenced directly on both strands with multiple primers as described previously (25). When identical sequences were obtained (suspicious of laboratory contamination) and to confirm sequence uncertainties such as frame shifts, we repeated the RNA isolation, RT-PCR, and subsequent sequencing with the original materials.

### Phylogenetic Analysis

Nucleotide sequences were aligned with the Clustal W program running within the Bioedit software package, version 5.0.9. Maximum likelihood trees were generated with the Seqboot and Dnaml packages of Phylip version 3.6 by using 100 bootstraps and 3 jumbles. The consensus tree was calculated by using the Consense package of Phylip 3.6 and was subsequently used as usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Finally, the trees were rerooted at midpoint by using the Retree software of Phylip 3.6. Trees were visualized with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9 (33). Sequences are available from GenBank under accession no. AY295930 to AY296012 (F partial) and AY304360 to AY304362 (complete F for NL/17/00, NL/1/99, and NL/1/94, respectively), AF371337 (complete genome NL/1/00), and AY296014 to AY296047 (complete G regions).

### Virus Preparations and Titrations

Viruses were isolated on tertiary monkey kidney (tMK) cells as previously described (25). For each genetic lineage a prototype virus isolate was chosen on the basis of its ability to grow to high titers on tMK cells and to reflect the specific genotype for the lineage. Virus titrations were cultured for 7 days, and infected wells were identified by immune fluorescence assays (IFA) with HMPV-specific polyclonal antiserum raised in guinea pigs. Titers were expressed in 50% tissue culture infectious dose (TCID<sub>50</sub>).

### Antisera

Lineage-specific polyclonal HMPV antisera were raised by infecting ferrets with 1 mL of virus-infected tMK supernatants containing approximately 10<sup>4</sup>–10<sup>5</sup> TCID<sub>50</sub> virus. All infections were performed in duplo, and the animals with the highest antibody responses are shown. Serum samples were collected at days 0 and 28 postinfection (ferret 1 and 2) or at days 0 and 21 (ferret 3 to 6). Infections were performed as follows: ferrets 1 and 3: HMPV NL/1/00, prototype virus for lineage A1. Ferrets 2 and 5: HMPV NL/1/99, prototype virus for lineage B1. Ferret 4: HMPV NL/17/00, prototype virus for lineage A2 and ferret 6: HMPV UK/5/01 a virus from lineage B2. Ferrets were housed in isolator cages to avoid cross-infections.

HMPV-specific polyclonal antisera were raised in guinea pigs as previously described (25). Antisera raised in separate guinea pigs against viruses from the two main genetic lineages (A and B) were mixed 1:1, and this mixture tested positive against all HMPV isolates in IFA.

### Virus Neutralization Assays

Virus neutralization assays of heat-inactivated (30 min 56°C) ferret serum samples were performed as previously

described (25). Briefly, twofold serial serum dilutions starting at 1:8 were incubated with approximately 30 TCID<sub>50</sub> virus. Seven days after infection of tMK cells with the antibody and virus mixture, IFA was performed with the guinea pig antiserum. The virus neutralization titer was defined as the reciprocal of the highest serum dilution at which no positive IFA signal was obtained (depicted as means of duplicate measurements). Each experiment included virus titrations of the working solution of the virus, using twofold dilutions, and 10–100 TCID<sub>50</sub> per well was considered acceptable.

## Results

### Variation in the Fusion Protein Gene

Partial F gene sequences (nucleotide [nt] 780–1,221 in the F ORF) were obtained from clinical samples collected from 84 HMPV-infected patients. Phylogenetic analysis of these sequences confirmed two main genetic lineages, A and B. Each of these lineages appeared to consist of two sublineages, which were tentatively named A1, A2, B1, and B2 (Figure 1A).

Comparison of the sequences showed high percentage identities between members of the same sublineage (nt: 97%–100%, amino acids [aa]: 99%–100%), members of the two different sublineages within each main lineage (nt: 94%–96%, aa: 97%–99%), and between members of the two different main lineages A and B (nt: 84%–86%, aa: 94%–97%). Whereas no specific amino acid residue substitutions could be found between sequences from subgroups A1 and A2, there were 5 specific aa substitutions between sequences from genotypes A and B, and one substitution between B1 and B2 (Table 1). The low variability was also observed when complete F protein genes from prototype viruses for each sublineage were sequenced (Figure 2).

### Variation in the Attachment Protein Gene

Nucleotide sequences of the region between the start codons of the G and the polymerase (L) reading frame were obtained for 35 samples. Phylogenetic analysis showed the same clustering of the sequences over the four sublineages as seen for the F protein gene (Figure 1B). The G region showed some variation in length, from 860 nt to 908 nt. The first 657–708 nt have been described as the putative primary G ORF (4). Alignment of the primary G ORFs showed a variation in length, even for members of the same sublineage, due to single nucleotide substitutions that resulted in premature termination codons (Figure 3). For two samples a change in ORF was observed as a result of an addition (BR/2/01: G at position 519) or a deletion (NL/2/93: C at position 243) of a single nucleotide. These mutations resulted in relatively short G ORFs (NL/2/93:

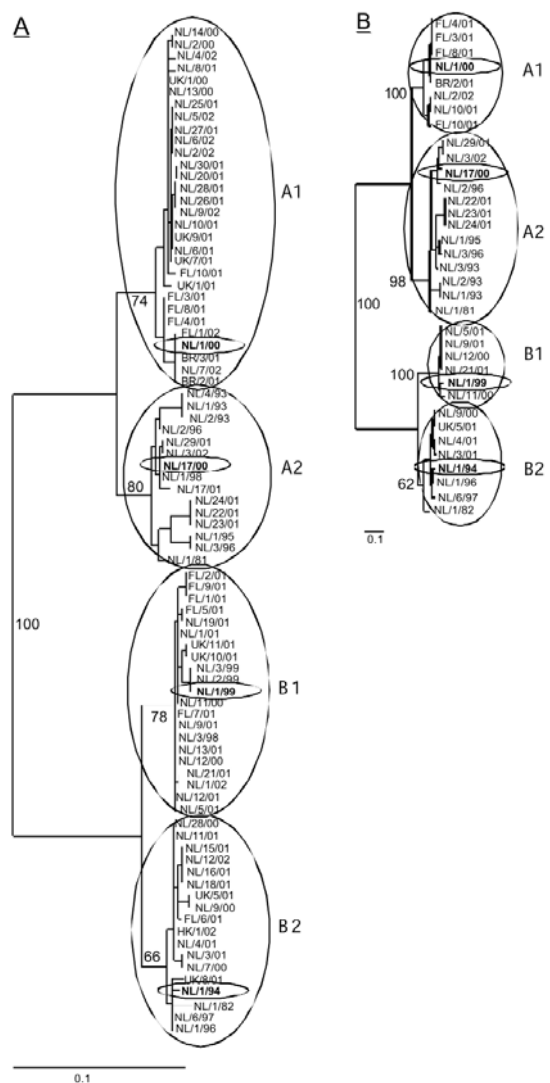


Figure 1. Phylogenetic trees constructed based on the (A) partial F gene (Open reading frame [ORF] position 780–1,221,  $n = 84$ ) or (B) the complete G coding region (start G ORF to start L ORF,  $n = 35$ ). Trees were generated by maximum likelihood analysis using 100 bootstraps and 3 jumbles. The scale representing the percentage of nucleotide changes is shown for each tree. Bootstrap values are based on the consensus trees, and relevant numbers are shown in the tree. The four prototype viruses are shown in bold-face, with ovals drawn around them. NL, viruses from the Netherlands; FN, viruses obtained from Finland; UK, viruses obtained from the United Kingdom; HK, viruses obtained from Hong Kong; BR, viruses obtained from Brazil.

110 aa; BR/2/01: 193 aa) because of premature termination and in drastic changes in the deduced amino acid sequences of the carboxy-terminus of the G proteins. Comparison of the primary G ORF sequences, excluding sequences of NL/2/93 and BR/2/01 because of the putative frame shifts, showed a relatively high percentage identity between members of the same sublineage (nt: 93%–100%,

Table 1. Lineage-specific amino acid substitutions between the four sublineages in the fusion open reading frame between position 260 and 407

Sublineage	aa <sub>286</sub>	aa <sub>296</sub>	aa <sub>312</sub>	aa <sub>348</sub>	aa <sub>404</sub>
A1	V	K	Q	K	N
A2	V	K	Q	K	N
B1	I	N	K	R	P
B2	I	D	K	R	P

aa: 75%–99.5%), less identity between members of the two different sublineages within each main lineage (nt: 76%–83%, aa: 60%–75%), and low sequence identity between members of the two different main lineages A and B (nt: 50%–57%, aa: 30%–37%).

The position of the hydrophobic domain, a high percentage of proline, serine, and threonine residues and a cysteine residue at position 27 are features shared by all HMPVs. Whereas the cytoplasmic tail was conserved among all members (58%–70% aa identity), the proposed ectodomains (start aa 51) were quite variable (18%–25% aa identity between lineage A and B). The number and position of potential sites for N-linked glycosylation sites varied even within each sublineage, from two to six potential sites, with one located at the proposed cytoplasmic tail conserved among all lineages.

**Geographic and Temporal Distribution**

Analysis of HMPV sequences obtained from samples received from different countries indicated that sequences from Finland, the United Kingdom, and the limited sequences from Asia and South America, were found on branches between the Dutch sequences in the F tree, and not as a separate lineage. The variation between sequences obtained from samples from a single country was found in the same range as the variation found between samples obtained from different countries. In agreement with the genetic lineages of HMPV observed worldwide, which usually includes sequences similar to those of isolate NL/1/00 or NL/1/99, geographic clustering does not appear to apply to HMPV.

The different HMPV samples were obtained during the last 20 years with most from 2000 to 2002 and 14 in the 1990s. As indicator for possible fixation of amino acid variation over time, we analyzed the G ORF amino acid sequence of members in sublineage A2 and B1 (containing samples from 1981 to 2002) in more detail. The amino acid sequence variation between the viruses from 1981 and 2001 was in the same range as the variation found between viruses from 2001, and alignments of the sequences did not indicate fixation of amino acid changes between 1981 and 2002. Thus, antigenic drift, as observed for influenza A and B viruses, does not appear to be an important phenomenon for HMPV.

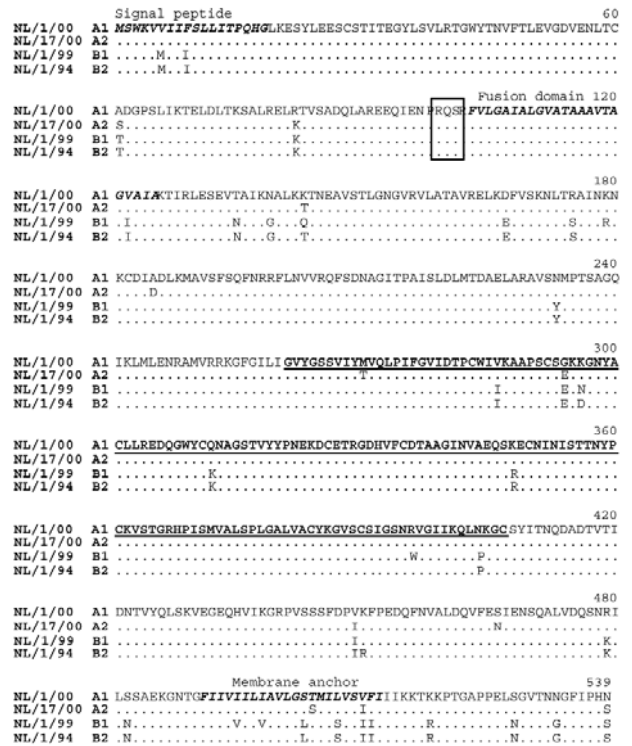


Figure 2. Amino acid sequence comparison of the fusion protein genes of prototype human metapneumovirus isolates of each sublineage. The predicted signal peptide, fusion domain, and membrane anchor are shown in italics in boldface type, the cleavage sites are boxed, and the region sequenced for 84 samples is underlined in boldface type. Periods indicate the position of identical amino acid residues relative to isolate NL/1/00.

**Antigenic Variation**

To address the antigenic variation between the genetic lineages A and B, we raised antisera in ferrets against isolate NL/1/00, the prototype virus for lineage A1, and against isolate NL/1/99, the prototype virus for lineage B1. The serum samples were collected 28 days postinfection and tested in virus neutralization assays against the homologous and heterologous viruses. In three independent experiments, the virus titer used per well varied from 10 to 50 TCID<sub>50</sub>; this variation did not affect the measured virus neutralization titers (Table 2). Ferret 1, infected with the lineage A prototype virus (NL/1/00), showed a 48- to 128-fold higher virus neutralization titer against the homologous virus NL/1/00 than to the heterologous virus NL/1/99. Similarly, ferret 2, infected with the lineage B prototype virus NL/1/99, had a 16- to 96-fold higher homologous than heterologous virus neutralization titer.

In a second experiment, ferret antisera were raised to viruses from all four sublineages. To measure the most specific serologic response, serum samples were collected 21 days postinfection, after which homologous and heterologous virus neutralization titers were measured

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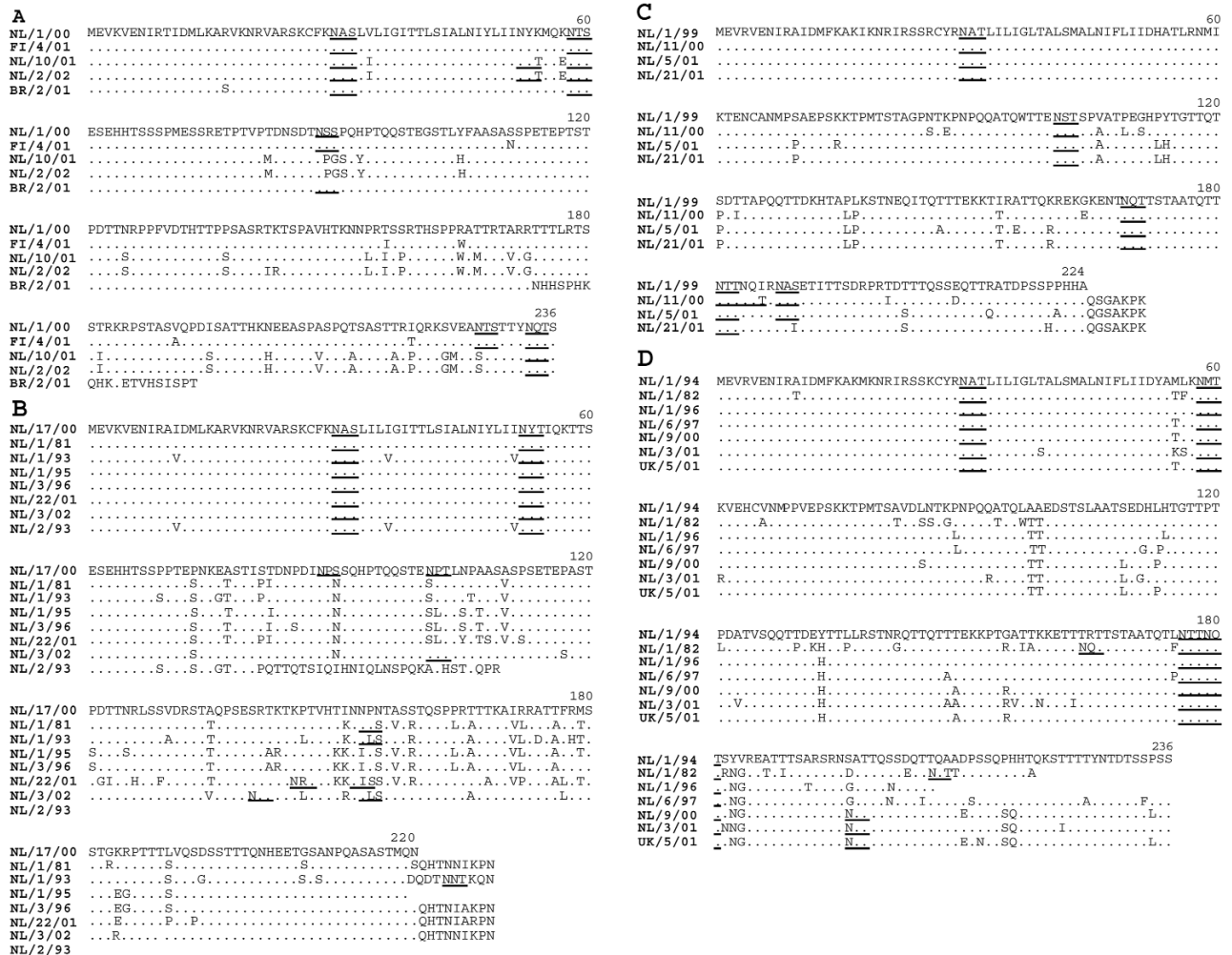


Figure 3. Amino acid sequence comparison of the putative attachment (G) protein of human metapneumovirus strains per genetic sub-lineage; A1 (A), A2 (B), B1 (C), and B2 (D). For each sublineage only representative samples are depicted, resulting in 24 sequences. Representative sequences were chosen, so that from each year in which samples were obtained at least one sequence is depicted, and sequences with only a few amino acid substitutions were omitted. Potential N-linked glycosylation sites are underlined, and periods indicate the position of identical amino acid residues relative to the first sequence in each subgroup. Numbers indicate the nucleotide position in the primary G Open reading frames.

(Table 3). Within each main genetic lineage, a high degree of cross-neutralization was observed between viruses from the two sublineages (e.g., A1 vs. A2 and B1 vs. B2), which is reflected in the low ratio between homologous to heterologous virus neutralization titer (0.5 to 3.0). Although serum samples from ferrets 3 to 6 had slightly lower homologous virus neutralization titers than those of ferrets 1 and 2, serum samples raised against viruses from the main lineage A still showed a 12- to 24-fold higher virus neutralization titer against the lineage A viruses than to lineage B viruses. Similarly, serum samples raised against viruses from lineage B had a 16- to 43-fold higher virus neutralization titer against the lineage B viruses than to lineage A viruses.

Discussion

In this study, the genetic heterogeneity of HMPV was addressed by analysis of the nucleotide and predicted amino acid sequences of part of the F (n = 84), complete F (n = 4), and the complete G (n = 35) protein genes. Phylogenetic analysis of these sequences showed two main lineages (A and B) with each divided into two sublineages (1 and 2). As was described for HRSV and APV, the F protein was highly conserved, which is in agreement with F proteins of pneumoviruses having structural and functional constraints for amino acid mutations (34). On the basis of the high percentage sequence identity for the complete F proteins of the prototype viruses of the four lineages, sequences for the complete F proteins of all 84 sam-



Table 2. Homologous and heterologous virus neutralizing antibody titers of serum samples obtained from ferrets infected with HMPV viruses belonging to different genetic sublineages<sup>a,b</sup>

Experiment	Virus used in virus neutralization	TCID <sub>50</sub> /well	Ferret 1 NL/1/00 [A1]	Ferret 2 NL/1/99 [B1]
1	NL/1/00	12	<b>1,024</b>	32
	NL/1/99	9	16	<b>512</b>
2	NL/1/00	30	<b>1,024</b>	8
	NL/1/99	20	8	<b>768</b>
3	NL/1/00	40	<b>768</b>	24
	NL/1/99	25	16	<b>768</b>
Ratio A–B			48–128	
Ratio B–A				16–96

<sup>a</sup>Virus neutralizing antibody titers obtained in three independent experiments for serum samples collected 28 days postinfection from ferrets 1 and ferret 2 (infected with NL/1/00 and NL/1/99 respectively). TCID<sub>50</sub>, 50% tissue culture infectious dose; HMPV, human metapneumovirus.

<sup>b</sup>Homologous virus neutralization titers are bold. Values are average of duplicate measurements. Ratios are given as the homologous virus neutralization titers divided by the heterologous virus neutralization titers.

ples would probably demonstrate similar low variability. In contrast to the F protein, the nucleotide and predicted amino acid sequences of the complete G coding regions showed high sequence diversity (as low as 30%–37% aa identity). Besides the high amino acid sequence variation, we observed variation in length of the different G proteins. Where most of the length variation was due to nucleotide substitutions, two of the samples showed a change in reading frame due to deletion or addition of single nucleotides. Frame shift mutations and use of alternative reading frames have been described for HRSV (35–37). As described for isolate NL/1/00, the G coding region of the 35 samples sequenced in the present work indicated long alternative ORFs. However, these secondary ORFs varied in length and position compared to the ones described (4). Whether premature stop codons, the incidence of frame shift mutations, and possible use of alternative reading frames influence the antigenic properties of the viruses needs to be examined in more detail.

Phylogenetic analyses showed that the HMPV samples obtained from different years and from different countries were randomly distributed over all four sublineages. For HRSV it has also been reported that very similar viruses were isolated at different times and from geographically distant sites (36). Different lineages within HRSV subgroup A and B have been found on the basis of the variation in the G protein. Within each subgroup, progressive

accumulation of amino acid changes was noted, suggesting that the G protein of HRSV might be susceptible to immune pressure (36). Analysis of the amino acid sequences of the HMPV samples described in this study did not indicate such accumulation over time. However the following observations indicate that the variation of the HMPV G protein might occur as a result of immunogenic pressure in a same manner as was postulated for the RSV G protein: 1) most of the amino acid sequence variation was found in the extracellular domain of the G protein, 2) the variation found at the amino acid sequence level was higher than that at the nucleotide sequence level, 3) the number and position of potential glycosylation sites were not conserved, and 4) deletions, additions and substitutions of single nucleotides resulted in premature stop codons and drastic changes of the carboxy terminal of the protein (18,36). Until a larger number of more chronologically diverse HMPV samples have been examined, this issue remains inconclusive.

To address the antigenic relationship between members of the different HMPV lineages, we tested ferret sera raised against viruses from the four sublineages in virus neutralization assays. Serologic responses upon infections tend to broaden over time. On the basis of the relatively close genetic relationship between sublineages A1 and A2 or B1 and B2, we decided to collect serum samples at an early time point, to obtain large antigenic differences

Table 3. Homologous and heterologous virus neutralizing antibody titers of sera obtained from ferrets infected with HMPV viruses belonging to different genetic sublineages<sup>a,b</sup>

Virus used in virus neutralization	Ferret 3 NL/1/00 [A1]	Ferret 4 NL/17/00 [A2]	Ferret 5 NL/1/99 [B1]	Ferret 6 UK/5/01 [B2]
NL/ 1/00	<b>256</b>	256	6	32
NL/17/00	512	<b>768</b>	12	24
NL/ 1/99	16	32	<b>256</b>	384
UK/ 5/01	12	64	256	<b>512</b>
Ratio A–B		16–21		
Ratio B–A			21–43	16–21

<sup>a</sup>Virus neutralizing antibody titers obtained for sera collected 21 days post infection from ferrets 3 to 6 (infected with NL/1/00, NL/17/00, NL/1/99 and UK/5/01, respectively). HMPV, human metapneumovirus.

<sup>b</sup>Homologous virus neutralization titers are bold. Values are average of duplicate measurements. Ratios are given as the homologous virus neutralization titers divided by the heterologous virus neutralization titers.

between the four sublineages. The low homologous virus neutralization titers in serum samples collected 21 days postinfection may explain the lower ratio between homologous and heterologous virus neutralization titers as compared to sera collected 28 days postinfection. The studies with serum samples collected at 21 days postinfection showed that viruses within one main lineage (e.g., A1 and A2 or B1 and B2) were antigenically closely related. The difference in homologous and heterologous virus neutralization titers between members of the two different lineages A and B titers (12- to 128-fold higher homologous titer than heterologous titer) indicate a difference in antigenicity between lineage A and B. Classic virology studies have used a definition of a homologous-to-heterologous virus neutralization titer ratio of >16 for defining serotypes. This same definition notes that if neutralization shows a certain degree of cross-reaction between two viruses in either or both directions (homologous-to-heterologous titer ratio of 8 or 16), distinctiveness of serotype is assumed if substantial differences in sequences are observed (29). On the basis of our results, and based on the described definition, we propose defining the two main lineages of HMPV as serotypes A and B. The HMPV samples were obtained from different study populations, from different countries, and from patients with a wide spectrum of clinical signs. So far, we have no indication of an association between infection with either of the serotypes and a specific study group or with severity of disease. More epidemiologic studies are needed to address this issue.

The circulation of two serotypes of HMPV might have implications for the development of vaccines. Studies in cynomolgous macaques showed that reinfection is suppressed by high titers of virus neutralization antibodies against the homologous virus and far less by heterologous virus neutralization antibodies (data not shown). So far, one heterologous reinfection has been reported in humans (28). However, children approximately  $\geq 5$  years of age have higher virus neutralization antibody titers than those 1–2 years of age (25), which suggests that reinfections may occur frequently, most likely with the viruses from the heterologous serotype. For RSV, the importance of difference in antigenicity between the two subgroups regarding protective immunity and vaccine development is still a subject of discussion. However, in animals and humans, the neutralizing capacity against homologous viruses is higher than that against heterologous viruses, and in animals high homologous virus neutralization titers protect against reinfection. In humans, reinfection often occurs with a strain from the heterologous group, and high homologous virus neutralization antibody titers protect against severe infection (13). The two serotypes of HMPV might resemble the two subgroups of HRSV in immunogenic properties, although more extensive epidemiologic and

immunologic studies have to prove this. The cross-reactive immunity provided by the F protein may be sufficient to overcome the effects of changes in the G protein. For HRSV the immune response against the F protein is cross-reactive between subgroup A and B, whereas the response against the G protein is subgroup (and sometimes even genotype) specific (14,16,38). The prophylactic use of a virus neutralization monoclonal antibody preparation directed against the HRSV-F protein has been shown to decrease the severity of lower respiratory tract diseases caused by both subgroups of RSV (39–41). In a similar way, the conserved F protein of HMPV could be a target for the development of monoclonal antibodies for treatment of HMPV-infected persons.

Our data support a technical description of two serotypes of HMPV in experimentally infected ferrets. The existence and relevance of these serotypes in other animal species, including humans, has yet to be determined.

Our results in combination with data published by others (26,27,42) demonstrate that HMPV clusters in two globally distributed serotypes. However, the identification of two serotypes does not exclude the possible existence of more serotypes or sublineages. The described viruses were all identified by using primers against conserved regions in the genome of the four prototype viruses, but in order to allow identification of more diverse HMPV strains, virus isolation of original materials is a standard procedure in our laboratory.

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Ms. van den Hoogen is a graduate student at the virology department of Erasmus Medical Center in Rotterdam, the Netherlands, where she studies the prevalence, clinical impact, and pathogenesis of human metapneumovirus infections.

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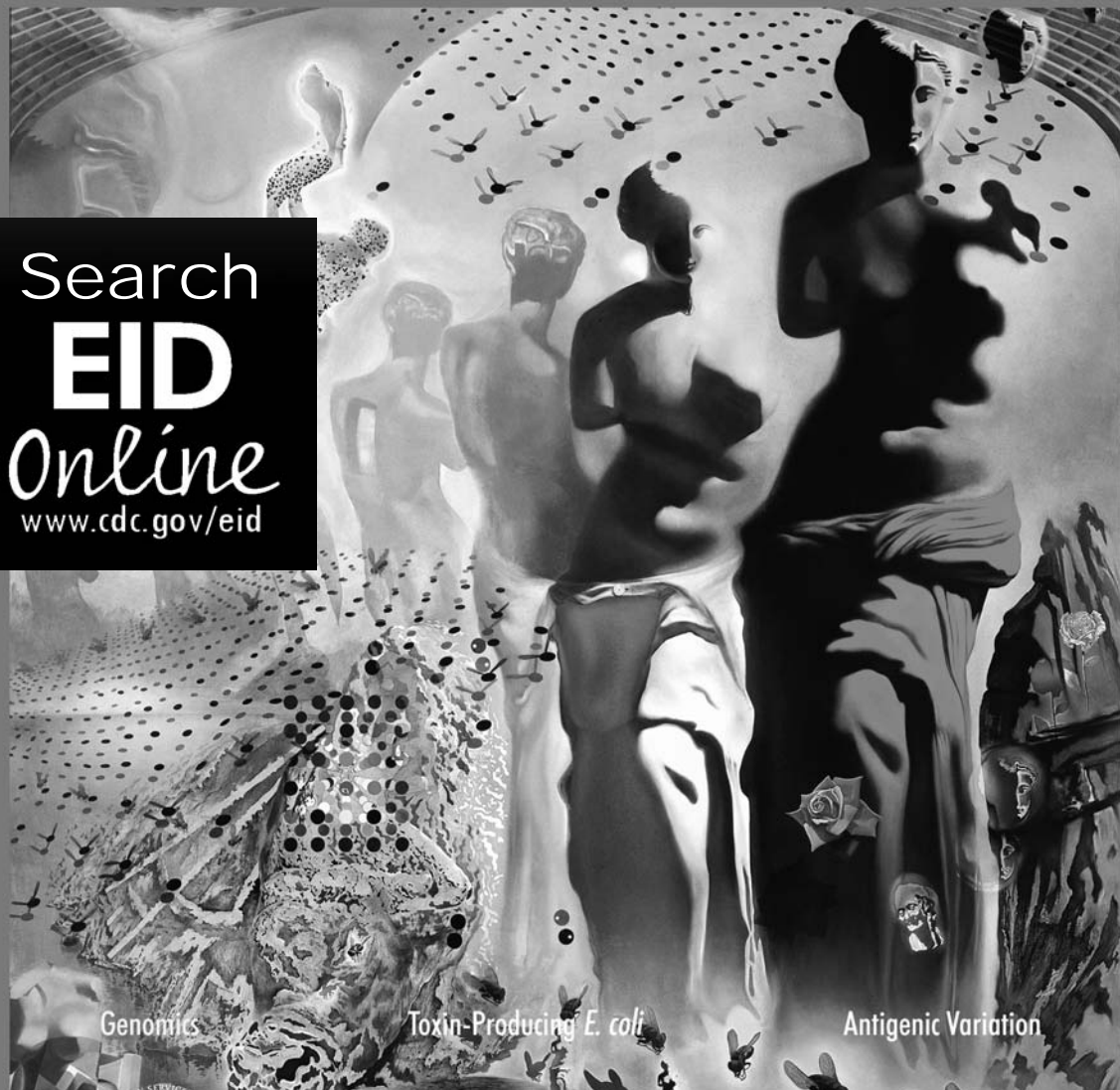
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# *Pneumocystis jirovecii* Dihydropteroate Synthase Genotypes in Immunocompetent Infants and Immunosuppressed Adults, Amiens, France

Anne Totet,\* Sophie Latouche,† Philippe Lacube,† Jean-Claude Pautard,\* Vincent Jounieaux,\*  
Christian Raccurt,\* Patricia Roux,† and Gilles Nevez\*

To date, investigations of *Pneumocystis jirovecii* circulation in the human reservoir through the dihydropteroate synthase (DHPS) locus analysis have only been conducted by examining *P. jirovecii* isolates from immunosuppressed patients with *Pneumocystis* pneumonia (PCP). Our study identifies *P. jirovecii* genotypes at this locus in 33 immunocompetent infants colonized with *P. jirovecii* contemporaneously with a bronchiolitis episode and in 13 adults with PCP; both groups of patients were monitored in Amiens, France. The results have pointed out identical features of *P. jirovecii* DHPS genotypes in the two groups, suggesting that in these groups, transmission cycles of *P. jirovecii* infections are linked. If these two groups represent sentinel populations for *P. jirovecii* infections, our results suggest that all persons parasitized by *P. jirovecii*, whatever their risk factor for infection and the form of parasitism they have, act as interwoven circulation networks of *P. jirovecii*.

Dihydropteroate synthase (DHPS) is the enzymatic target of sulfonamides, which are the major drugs for *Pneumocystis* pneumonia (PCP) prophylaxis or treatment (1). *Pneumocystis jirovecii* (human-specific *Pneumocystis*) organisms with nonsynonymous mutations at nucleotide positions 165 and 171 on the DHPS locus have been detected in HIV-infected patients with PCP who had previously been treated with sulfonamides (2–14). Prior exposure to sulfonamide drugs has been identified as a predictor of mutant genotypes (2–12). In addition, the city of patient residence has also been identified as an independent risk factor (6,8), a factor that supports the hypothesis that *P. jirovecii* is transmitted from infected treated patients to susceptible untreated patients, either directly or

through a common environmental source. The analysis of *P. jirovecii* DHPS locus may thus serve as a useful circulation marker of the microorganism in the human reservoir.

To date, investigations of *P. jirovecii* circulation through the DHPS locus analysis have only been performed by examining isolates from immunosuppressed adults with PCP (8,15,16). However, *Pneumocystis* infections may cover a wide spectrum of clinical signs and symptoms; colonization with *Pneumocystis* in immunocompetent infants at risk for primary infection may constitute a large part of this spectrum (17,18). Genotyping of *P. jirovecii* at the internal transcribed spacer (ITS) locus showed that these infants were infected with similar genotypes as those previously reported in compromised hosts with PCP; this similarity is compatible with the hypothesis that both groups of patients make up a common human reservoir for the fungus (19).

The existence of similar genomic characteristics at another locus, in particular at the DHPS locus, among *P. jirovecii* isolates from these two groups would provide additional arguments in favor of the fungus' circulating within a reservoir made up of persons with different clinical forms of *P. jirovecii* infection. For these reasons, we retrospectively investigated for DHPS genotyping archival *P. jirovecii* isolates from immunocompetent infants colonized with *P. jirovecii* and from immunocompromised adults with PCP. Both groups of patients lived in the same French city. The results of this study were reported in part in a conference report (20).

## Materials and Methods

A total of 58 archival *P. jirovecii* isolates obtained from 58 patients (45 infants and 13 adults) were retrospectively

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analyzed for DHPS genotyping. All of these patients were monitored in the same University Hospital in Amiens, France.

Forty-five archival nasopharyngeal aspirates (NPA) obtained from 45 nonpremature, immunocompetent infants (median age 4.3 months [range 1.9–11.8]; sex ratio 26 boys and 19 girls) were examined. The 45 infants were hospitalized in the period from November 1999 to April 2001. The specimens initially tested positive for *P. jirovecii* by a polymerase chain reaction (PCR) assay that amplifies a portion of the gene encoding the mitochondrial large sub-unit rRNA (mtLSUrRNA) (17). All infants initially had an acute respiratory syndrome compatible with a diagnosis of bronchiolitis and no patent immunodeficiency. The presence of *P. jirovecii* in these infants was considered to reflect merely a colonization. Indeed, clinical improvement was obtained with short-term hospitalization (1–12 days), despite the absence of specific treatment for the fungus. Furthermore, *P. jirovecii* was associated with the respiratory syncytial virus or with bacteria (*Moraxella catarrhalis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Bordetella pertussis*) in 35 of 45 infants. None had a past history of sulfonamide treatment. The infants' characteristics are summarized in Table 1.

Thirteen archival bronchoalveolar lavage (BAL) specimens obtained from 13 immunosuppressed adults in whom PCP was diagnosed were also examined. The 13 patients were hospitalized in the period from June 1996 to November 2001. The specimens initially tested positive for *P. jirovecii* by microscopy examination that used methanol-Giemsa stain and an immunofluorescence assay (MonofluoKit *Pneumocystis*; Bio-RAD, Marnes la Coquette, France), and by the PCR at mtLSUrRNA. The underlying conditions were HIV infection (nine patients), renal transplantation (two patients), and long-term corticosteroid treatment for systemic lupus erythematosus (one patient) and for hepatic granulomatosis (one patient). None of the patients had *P. jirovecii* prophylaxis with sulfon-

amide drugs in the 3 months preceding the BAL retrieval. The patients' characteristics are summarized in Table 1. DNAs extracted from NPA and BAL were stored at  $-20^{\circ}\text{C}$  until they were typed.

The *P. jirovecii* DHPS locus was analyzed by PCR–restriction fragment length polymorphism (RFLP). The DHPS sequence was first amplified by a nested PCR assay. The two rounds of PCR were performed under the same conditions. Each reaction mixture contained the following reagents at the indicated final concentrations: 10 mmol/L Tris-HCl (pH 8.8), 0.1% Tween 20 (vol/vol), 2.5 mmol/L MgCl<sub>2</sub>, 200 μmol each deoxynucleoside triphosphate (dNTP set, Eurogentec, Seraing, Belgium), 0.6 μmol/L each oligonucleotide primer, and 0.02 U DNA polymerase (Red Goldstar DNA polymerase, Eurogentec)/μL. The first PCR round was conducted with primer pair A<sub>HUM</sub> (5'- GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C-3') and B<sub>HUM</sub> (5'- CAT AAA CAT CAT GAA CCC G -3') (14) by using a "touch-down" program. In the first cycle, the denaturation step was 92°C for 30 s, the annealing step was 52°C for 1 min, and the extension step was 72°C for 1 min. This cycle was repeated 10 times but with each annealing step at 1°C lower temperature than the preceding cycle. Subsequently, the last cycle, with an annealing at 42°C, was repeated 20 times. The second PCR round was performed with primer pair C<sub>PRIM</sub> (5'- CCC CCA CTT ATA TCA-3') and D<sub>PRIM</sub> (5'- GGG GGT GTT CAT TCA -3') (21), for 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The PCR products from the first and the second rounds underwent electrophoresis on a 1.5% agarose gel containing ethidium bromide to visualize the expected bands of 766 bp and 269 bp, respectively. To avoid contamination, each step (reagent preparation, extraction, and amplification) was performed in different rooms with different sets of micropipettes and using barrier tips. PCR mixtures and the extraction step were prepared in a laminar-flow cabinet. Rooms required for

Table 1. Characteristics of 45 infants and 13 adults from the University Hospital of Amiens, France, for whom *Pneumocystis jirovecii* organisms were examined at the dihydropteroate synthase locus

Characteristics	Infants	Adults
Risk factor for <i>Pneumocystis</i> infection	Young age <sup>a</sup>	Immunodeficiency <sup>b</sup>
No. of patients	45	13
Median age (range)	4.3 mo (1.9–11.8)	35 y (29–67)
Sex ratio, M/F	26/19	10/3
Period of specimen retrieval	November 1999–April 2001	June 1996–November 2001
Kind of specimens	Nasopharyngeal aspirate	Bronchoalveolar lavage
Method of <i>P. jirovecii</i> detection	PCR assay <sup>c</sup>	Both microscopy <sup>d</sup> and PCR assay
Form of <i>Pneumocystis</i> infection	Colonization <sup>e</sup>	<i>Pneumocystis</i> pneumonia

<sup>a</sup>Young age renders it compatible with primary *Pneumocystis* infection.

<sup>b</sup>HIV infection (n = 9), renal transplant (n = 2), and long-term corticosteroid treatment (n = 2).

<sup>c</sup>PCR, polymerase chain reaction assay, at the mitochondrial large sub-unit rRNA gene.

<sup>d</sup>Methanol-Giemsa stain and immunofluorescence assay (Bio-Rad, Marnes la Coquette, France).

<sup>e</sup>Clinical improvement observed despite the absence of treatment for the fungus.

amplified DNA manipulation were continuously submitted to an airflow with UV decontamination (SPRW 30 GR4; Paragerm, Inc., Paris, France).

To monitor for possible contamination, a negative control (ultrapure distilled water) was included in each PCR step. The RFLP assay was performed with two restriction enzymes, according to the manufacturer's recommendations (Promega Corporation, Madison, WI). One part of the nested PCR products was digested with the restriction enzyme *AccI*, and another part with *HaeIII*, which make possible the detection of mutations at nucleotide positions 165 and 171, respectively (5). The restriction profiles were visualized by electrophoresis of each digested product on a 1.5% agarose gel with ethidium bromide, as described in Figure 1. The mutations inhibit the restriction enzyme activity. Thus, a wild genotype was shown, after digestion with *AccI*, by two fragments of 181 bp and 88 bp, and after digestion with *HaeIII*, by two other fragments of 173 bp and 96 bp. A mutant genotype that has a mutation at nucleotide position 165 (change from A to G, corresponding to a change from Thr to Ala at aminoacid position 55) was shown, after digestion with *AccI*, by only one uncut fragment of 269 bp, and after digestion with *HaeIII*, by the two fragments of 173 bp and 96 bp. A mutant genotype that has a mutation at nucleotide position 171 (change from C to T, corresponding to a change from Pro to Ser at aminoacid position 57) was shown after digestion with *AccI*, by the two fragments of 181 bp and 88 bp, and after digestion with *HaeIII*, by only one uncut fragment of 269 bp. A double mutant genotype, which has mutations at nucleotide positions 165 and 171, was shown, after digestion with either *AccI* or *HaeIII*, by an uncut fragment of 269 bp.

## Results

The amplification of *P. jirovecii* DNA by using the DHPS-based PCR assay was positive for 33 of 45 NPA from infants that initially tested positive with the PCR directed at the mtLSUrRNA gene, whereas it was successful for 13 of 13 BAL specimens from adults. In each positive specimen with the DHPS-based PCR assay, the RFLP technique led to identification of a wild *P. jirovecii* DHPS genotype. However, mixed infections were diagnosed in three infants and one adult. Indeed, in three (9%) of the 33 NPA from infants, the wild genotype was associated with a mutant genotype. In one infant, the mutant genotype had a mutation at nucleotide position 165, whereas in two infants, it had a mutation at nucleotide position 171. In one (8%) of the 13 BAL from adults, the wild genotype was also associated with a mutant genotype, which had a mutation at nucleotide position 171. No infants or adults were infected with a mutant genotype either singly or with a double mutant genotype. The results are detailed in Table 2.

## Discussion

Most studies on *P. jirovecii* DHPS genotyping have focused on the relationship between *P. jirovecii* DHPS mutants and prior sulfonamide exposure on the one hand, and PCP outcome on the other hand (2–13,22–24). We have used the DHPS locus analysis differently, as a marker for studying the potential circulation of the fungus in the human reservoir, as it was recently used by Beard et al. and Huang et al. (8,15,16). Although a multilocus genotyping was recently reported as an efficient system for *P. jirovecii* characterization (25), in this study, we only analyzed the DHPS locus because it still remains the sole marker of circulation. We also obtained the first data on the analysis of *P. jirovecii* DHPS locus in immunocompetent infants at risk for primary *Pneumocystis* infection.

The first step of this analysis required a PCR assay. The amplification failed to give positive results for 12 of the 45 NPA from infants who initially tested positive for *P. jirovecii* by using the PCR at mtLSUrRNA. This difference in sensitivity between the two PCR assays can be explained by the fact the mtLSUrRNA gene is present in many copies within each *P. jirovecii* genome, whereas the folic acid synthesis gene, encoding the DHPS, is thought to

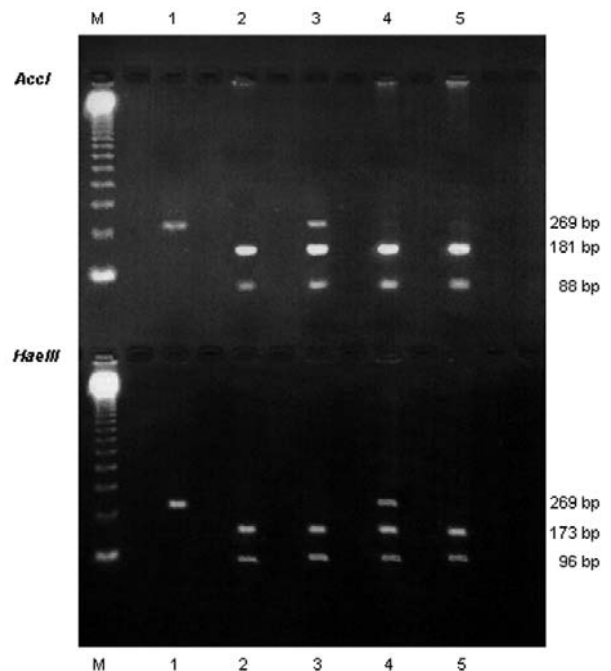


Figure 1. Identification of *Pneumocystis jirovecii* dihydropteroate synthase (DHPS) genotypes. Electrophoresis on 1.5% agarose gel of DHPS polymerase chain reaction products after digestion with *AccI* (upper line) and *HaeIII* (lower line). M, molecular weight marker (123 Gibco BRL; Cergy Pontoise, France). Lane 1: double mutant genotype; lanes 2 and 5: wild genotype; lane 3: mixed infection with a wild genotype and a mutant genotype that has the mutation at nucleotide position 165; lane 4: mixed infection with a wild genotype and a mutant genotype which has the mutation at nucleotide position 171.

Table 2. *Pneumocystis jirovecii* dihydropteroate synthase (DHPS) genotypes in immunocompetent infants with colonization and in immunosuppressed adults with *Pneumocystis pneumonia*, University Hospital of Amiens, France

DHPS genotype	Nucleotide at position (amino acid at position)		Infants	Adults
	165 (55)	171 (57)		
Wild genotype <sup>a</sup>	A (Thr)	C (Pro)	30	12
Double mutant genotype <sup>a</sup>	G (Ala)	T (Ser)	– <sup>b</sup>	–
Wild genotype + mutant genotype <sup>c</sup>	A/G (Thr/Ala)	C (Pro)	1	–
Wild genotype + mutant genotype <sup>d</sup>	A (Thr)	C/T (Pro/Ser)	2	1
Wild genotype + double mutant genotype <sup>e</sup>	A/G (Thr/Ala)	C/T (Pro/Ser)	–	–
Undetermined genotype	–	–	12 <sup>f</sup>	–

<sup>a</sup>Infection with a single genotype.  
<sup>b</sup>–, absence.  
<sup>c,d,e</sup>Mixed infections related to the association of a wild genotype with a mutant genotype.  
<sup>c</sup>Mutant genotype with a mutation at nucleotide position 165 (amino acid position 55).  
<sup>d</sup>Mutant genotype with a mutation at nucleotide position 171 (amino acid position 57).  
<sup>e</sup>Double mutant with both mutations at nucleotide positions 165 and 171.  
<sup>f</sup>Absence of DHPS amplification despite initial detection by polymerase chain reaction at mtLSUrRNA.

be present in only one copy (26). This difference is particularly manifest on specimens collected by noninvasive means, such as NPA, in which the amount of *P. jirovecii* is usually low. Indeed, NPA essentially recover cells from the upper respiratory tract, whereas the fungus primarily infects the alveolar spaces. Despite these difficulties, the identification of *P. jirovecii* DHPS genotypes was successful for three fourths of the samples we examined.

Most investigations of mutations on the *P. jirovecii* DHPS locus have used the direct sequencing of PCR products (3,4,6–8,10,11,14–16,22,23). More recently, a single-strand conformation polymorphism assay has been described as an alternative method for detecting DHPS mutations (12,27). We used the RFLP assay in this study since this method has a lower cost, is less time-consuming (5,28), and is more efficient for detecting mixed infections than direct sequencing (L. Diop Santos, pers. comm.). The use of restriction enzymes *AccI* and *HaeIII* for the digestion of the PCR products showed two mutations at nucleotide positions 165 and 171, as described above. The RFLP assay of *P. jirovecii* DHPS gene was assessed by Helweg-Larsen et al., who have examined 27 BAL specimens containing a mixture of wild and mutant DHPS genotypes, previously determined by direct sequencing (28). For detecting mutations at nucleotide positions 165 and 171 on *P. jirovecii* DHPS sequence, these researchers found a 100% concordance between DHPS genotypes determined by *AccI* and *HaeIII* restriction enzyme cleavage and by sequencing. Thus, the RFLP assay appears to be a reliable method for discriminating wild and mutant DHPS genotypes.

Mutations at nucleotide positions 165 and 171 have been correlated with prior sulfonamide treatment or prophylaxis (2–12). In our study, since none of the infants or adults had this medical history, the presence of *P. jirovecii* DHPS mutants has to be discussed. Because of the young age and, consequently, the short medical history of the infants, we could easily ensure that none had had prior

exposure to sulfonamides. Conversely, this exposure throughout the adults' lifetimes cannot strictly be ruled out. These difficulties have previously been raised by Huang et al., who have pointed out the need for a standardized definition of exposure to sulfonamides (29). In particular, the period during which sulfonamides have not been used, preceding patient sampling, to define the absence of selective pressure, varies according to the experience of each medical team. At any rate, in our study, no adults were treated with sulfonamides in the 3 months before BAL retrieval. In this group of patients, we detected *P. jirovecii* DHPS mutants with a frequency of 8%. This finding may reflect a basic level of infections caused by mutants in the absence of direct selective pressure; their presence is related to an incidental acquisition of the microorganism from humans treated with sulfonamides, either directly or through hypothetical environmental stages. In the same way, this hypothesis may explain the presence of DHPS mutants in the infant group.

Airborne transmission of the fungus from host to host has been demonstrated in rodent models (30), and several observations suggest that interindividual transmission occurs in humans (31,32). Moreover, *Pneumocystis* organisms infecting each mammalian species are host-specific, and the hypothesis of an animal reservoir for *P. jirovecii* can be excluded (33). Although an exosaprophytic form of the fungus cannot be ruled out, these data point toward the potential for the specific host to serve as its own reservoir and for PCP in humans as an anthroponosis with humans as a reservoir for *P. jirovecii*.

The 8% frequency with which we have detected mutants in PCP patients from Amiens who had no sulfonamide exposure is close to the figure reported for a similar group of patients in Milan, Italy (4% [2]) and in Copenhagen (10.5% [4]), while it appears lower than the rate in Rome (17% [11]), Tokyo (25% [23]), and various U.S. cities (15%–81% [3,6–8,16]). In France, data on DHPS genotypes concern patients living in Paris or Lyon,



as recently reported by Latouche et al. and Nahimana et al., respectively (12,13). The frequency of mutants in PCP patients who had no prior sulfonamide treatment or prophylaxis reaches 25% in Paris (13), and 29% in Lyon (12). The low proportion of mutants in Amiens in comparison to Paris and Lyon may be related to different features of *P. jirovecii* epidemiology in these cities. Amiens (population 150,000) is characterized by a low incidence of AIDS and PCP (34). Conversely, in Paris and its suburbs, a megalopolis of 10 million people, the incidence of these two infections is 30 times as high (34). In Lyon, the second largest city in France, this incidence is 10 times higher than in Amiens (34). Consequently, use of sulfonamides is widespread in Paris and Lyon, favoring the emergence of mutants and provoking a high risk for incidental acquisition of these mutants, even in patients not directly exposed to sulfonamides. This hypothesis is strengthened by a recent report of Miller et al., concerning patients in London, which showed that the decrease of sulfonamide prophylaxis use, related to the introduction of high-active antiretroviral therapy since 1996 conversely generated a reduction of mutant DHPS genotypes in London (36% compared to 16%) (35). Available frequencies of mutants in PCP patients living in Europe who had no prior sulfonamide exposure are shown in Figure 2.

We detected mutants in immunocompetent infants colonized with *P. jirovecii* and in immunosuppressed adults with PCP with frequencies of 9% and 8%, respectively. Besides these similar frequencies, the most frequent *P. jirovecii* DHPS genotype was the wild genotype. Mutant genotypes have only been detected within mixed infections. On the whole, genomic characteristics of *P. jirovecii* organisms at the DHPS locus in the two patient populations living in the same city are similar. In the United States, Beard et al. observed different genomic features at this locus among *P. jirovecii* isolates from adults and deceased infants (36). For these reasons, those researchers suggested that transmission cycles for *P. jirovecii* infection in infants and adults were independent. However, whether the two individual groups lived in the same American city was not specified. Conversely, our results of genotyping based on DHPS locus analysis suggest that these transmission cycles are linked, the two patient groups being part of a common reservoir in which the fungus may circulate.

If one considers that both of these patient groups may represent sentinel populations for *P. jirovecii* infections, other persons infected with *P. jirovecii* may also be actively involved in the circulation of the fungus. Indeed, new detection tools such as PCR assays have shown that pulmonary colonization with *P. jirovecii* occurs in patients with diverse levels of immunodeficiency (37) and in immunocompetent patients with lung diseases (38,39).

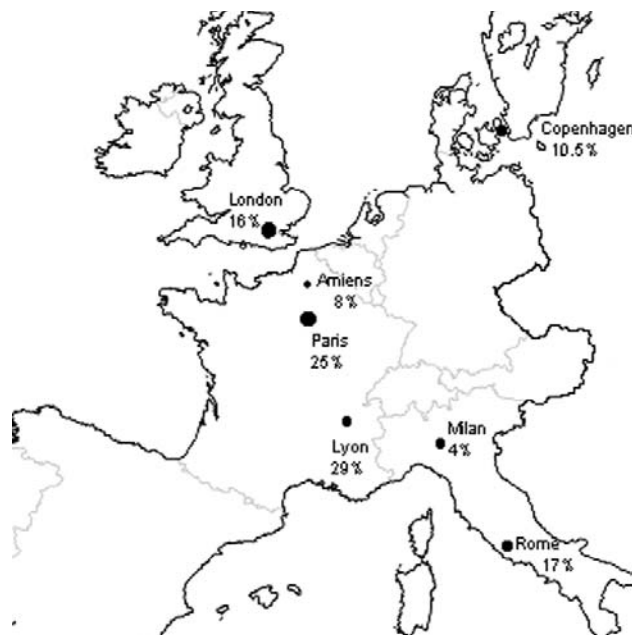


Figure 2. Frequencies of *Pneumocystis jirovecii* dihydropteroate synthase mutants in patients who had *Pneumocystis pneumonia* and no prior sulfonamide exposure, from diverse European cities. Amiens, France (the present study), Paris (13), Lyon (12), Copenhagen (4), Milan, Italy (2), Rome (11), and London (35).

Such assays have also shown that *P. jirovecii* can transiently parasitize immunocompetent healthcare workers after contacts with PCP patients (40). Our positive results of DHPS genotyping on specimens collected by noninvasive means (NPA) ensure further investigations of *P. jirovecii* circulation involving such populations, for whom invasive sampling cannot easily be performed. In fact, all parasitized persons, whatever their predisposition to *P. jirovecii* acquisition and the clinical form of *P. jirovecii* infection they have, may reflect a wide human reservoir of which all components are not yet characterized. New insights into the *P. jirovecii* reservoir could provide better prophylactic measures against *P. jirovecii* transmission and, consequently, PCP.

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Dr. Totet is a specialist in medical biology (Department of Parasitology, Mycology, and Travel Medicine, University Hospital of Amiens, France) with an advanced degree in parasitology. This study is a part of the work for her doctoral degree from the University of Picardy.

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# PorA Variable Regions of *Neisseria meningitidis*

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Subtypes, defined by variation in the outer membrane protein PorA, are an integral part of the characterization scheme for *Neisseria meningitidis*. Identification of these variants remains important as the PorA protein is a major immunogenic component of several meningococcal vaccines under development, and characteristics of PorA are used to provide detailed epidemiologic information. Historically, serosubtypes have been defined by reactivity with a set of monoclonal antibodies. However, nucleotide sequence analyses of *porA* genes have established that the panel of serosubtyping monoclonal antibodies is not exhaustive, and many *porA* variants cannot be detected. In addition, the nomenclature system used to define subtypes is inadequate. We examined all available nucleotide sequences of the *porA* VR1 and VR2 regions to identify and define subtype families. A revised nomenclature scheme, compatible with the previous serologic nomenclature scheme, was devised. A Web-accessible database describing this nomenclature and its relationship to previous schemes was established (available from: <http://neisseria.org/nm/typing/pora>).

*Neisseria meningitidis* is a major cause of bacterial meningitis and septicemia worldwide (1). In the absence of a comprehensive vaccine against this organism, the characterization of its variable surface antigens is important for epidemiologic monitoring and vaccine development (2). The serologic characterization scheme for meningococci comprises the following: groups, based on variants in the capsular polysaccharide; types, based on variants of the PorB outer membrane protein (OMP); subtypes, based on variants of the PorA OMP; and immunotypes, based on variants in the lipooligosaccharide (3). Within this scheme, PorA, also known as the class 1 OMP, is assigned the prefix "P1." followed by numbers, separated by commas, that correspond to the subtype designation (thus: P1.7,16). The two PorA variable regions (VR1 and VR2) that confer the subtypes are especially important

because they elicit bactericidal antibodies in humans (4). Consequently, a number of meningococcal vaccines under development contain the PorA protein as a major component (5).

Nucleotide sequence analyses of *porA* genes from multiple meningococcal isolates have established that the panel of serosubtyping monoclonal antibodies (MAbs) is not comprehensive. Meningococci are frequently only partially serosubtyped, and an increasing number of isolates are classified as non-serosubtypeable, either because a variant is not recognized by MAbs or because PorA is not expressed. This heterogeneous group of isolates can be fully characterized on the basis of their PorA VR1 and VR2 amino acid sequences deduced from nucleotide sequence data. To accommodate subtypes identified on the basis of sequence data alone, the scheme originally developed for MAb reactivity data (3) was modified so that VR families and variants were assigned on the basis of amino acid sequence relationships rather than their reactivity with specific MAbs. A distance matrix of all known VR1 and VR2 amino acid sequences was constructed, and VR amino acid sequences containing  $\geq 80\%$  identity to each other were grouped into VR families. The VR epitope recognized by an existing MAb raised against PorA, or the first defined amino acid sequence of a VR family, was arbitrarily designated as the prototype VR for that particular family. Successive distinct members of a VR family were designated as minor variants of that family, and as such were sequentially assigned an additional unique lower case letter, e.g., P1.5a, P1.5b, P1.5c (6).

Although this nomenclature system was sufficiently flexible to accommodate both novel subtypes determined from nucleotide sequence analyses and those defined by the reactivity of specific MAbs, limitations have become apparent. First, while the 80% similarity rule has generally proved adequate to assign VR families, it is open to misinterpretation, leading to the inappropriate designation of VR sequences. Second, the assignment of minor variants within VR families is limited by the number of letters in the alphabet (7,8). We present a revised nomenclature, which addresses these issues and shows the relationship of

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new designations to the previous designations and to the reactivities of the MAb panel. A database accessible through the Internet has been established, which will enable this scheme to be continually updated.

## Materials and Methods

### Bacterial Isolates

Two sets of meningococcal isolates were used for *porA* gene sequencing in this work. The first was a set of 393 isolates from cases of disease from diverse locations throughout the United Kingdom. These included 125 isolates from 1975; 100 isolates from 1985; 100 isolates from 1995; and 18 urethral isolates, provided by the Meningococcal Reference Unit, Manchester Public Health Laboratory, Manchester. Fifty isolates were provided by the Scottish Meningococcus and Pneumococcus Reference Laboratory, Glasgow. The second set of isolates included the 107 globally representative isolates obtained from both patients and carriers; these isolates were used to develop and evaluate the multilocus sequence typing isolate characterization scheme (9).

### *porA* Gene Sequences and Validation

Nucleotide sequences of *porA* genes encoding the variants included in Appendix Tables 1 and 2 (online only; available from: [http://www.cdc.gov/ncidod/EID/vol10no4/03-0247.htm\\_app.htm](http://www.cdc.gov/ncidod/EID/vol10no4/03-0247.htm_app.htm)) were obtained from the literature or GenBank, determined by sequencing of polymerase chain reaction (PCR) products from the above isolates, or submitted by personal communication or to the PorA Web site. Where possible, sequences not determined in this study were validated by requesting sequence electropherograms from depositors. When electropherograms could not be resolved, isolates were requested and the *porA* genes resequenced. Seven sequences contained errors on resequencing original isolates and were therefore removed from the new nomenclature scheme. The deposited VR sequences used in this study were those submitted to the PorA Web site by June 11, 2001.

### DNA Amplification and Nucleotide Sequence Determination of *porA*

Boiled meningococcal suspensions or DNA prepared from such suspensions with an Isoquick kit (Microprobe Corporation, Washington) were used as template to amplify the *porA* gene by using Taq Polymerase (Applied Biosystems, by Roche Molecular Systems Inc., Branchburg, NJ) with primers 210 and 211 (10). The amplification products were purified by precipitation with the addition of 0.6 V of 20% polyethylene glycol 8000/2.5M NaCl (11) and their nucleotide sequences determined at least once on each DNA strand. Sequence

reactions were carried out with primers 8L, 8U, 103L, 103U, 122L, 122U, 210, and 211 (10) using BigDye Ready Reaction Mix (Applied Biosystems) in accordance with the manufacturer's instructions. Unincorporated dye terminators were removed by precipitation of the termination products with the addition of 2.6 V of 96% ethanol and 115 mM sodium acetate. The reaction products were then separated and detected with an ABI Prism 377 automated DNA sequencer (PE Biosystems). Sequences were assembled from the resultant electropherograms with the STADEN suite of computer programs (12).

### Manipulation and Alignment of Sequences

Sequences were manipulated in SeqLab, part of the GCG software package (13). All unique nucleotide sequences for each VR were aligned with reference to both the nucleotide and the amino acid sequences, such that all sequences remained in frame, gaps were minimized, and similar codons were aligned.

### Identification of Families and Variants

To remain consistent with serologic and historical nomenclature, where a variant had been identified previously by serologic means, the identified sequence was used as a family prototype around which new sequences were grouped. An 80% amino acid identity cut-off—against the shortest sequence length when the sequences were of different length to allow for insertions, duplications, and deletions—was used as a guide in this grouping. In a few cases, a variant was assigned to a particular family even though the amino acid identity was slightly less than 80%, compared to the family prototype. In these cases, the new variant was still more similar to this particular family than to others but also contained a particular motif that was representative of family members. Therefore, a combination of overall similarity and presence of particular motifs was used to make the groupings. In a few cases, family-specific motifs were missing, but the sequences were otherwise identical or highly similar to members of the family. In such cases, the sequence was assigned as a variant of the family.

To further ensure that family groupings were consistent, the relationships among aligned nucleotide sequences encoding VR1 or VR2 were visualized by split decomposition analysis using SPLITSTREE version 3.1 (14). The split decomposition analysis was carried out in a sequential manner. In each analysis, a limited number of families were resolved, and the remaining variants were clustered together at a node. The variants that were resolved first were removed, and the analysis was repeated to resolve further families, and so on until all family groups were resolved (15). For analysis of the whole datasets, Hamming distances (equivalent to *p*-distance) were used

because some of the families were so diverse that using a substitution model was not possible. This method resolved the most distantly related families. The Kimura three parameter model (16) was used to determine whether related sequences constituted families.

A database and Web site containing all the assignments have been established (available from: <http://neisseria.org/nm/typing/pora>). The sequences are stored in a PostgreSQL database running on Linux. Perl scripts enable the database to be queried against either peptide or nucleotide sequences; when an identical match is not found, a BLAST search (17) can be performed to identify the nearest variant and family. Any length of sequence can be queried, enabling the variants to be quickly identified from a whole or partial gene sequence.

**Results**

**Validation of Sequence Variants**

The sequences defining the following subtypes in the previous nomenclature were not included in the new nomenclature as a consequence of the sequence validation: P1.2a, P1.2d, P1.5b, P1.10h, P1.10i, P1.10j, P1.18b, P1.19c, P1.24, P1.29.

**Resolution of VR Families**

The amino acid sequences of the prototype member of each of the VR families identified are shown aligned in Figures 1 and 2 together with corresponding nucleotide sequences. A total of 10 VR1 and 17 VR2 families were resolved. The most closely related VR families are VR2 P1.2 and VR2 P1.10, although the family prototypes are recognized by specific MABs that are not cross-reactive. Both families start with a consensus amino acid sequence of HFVQQ and end with PTLVP. They can be differentiated, however, by split decomposition where they cluster separately (15) and by certain motifs in their sequences. The P1.10 family members have a consensus motif QNKQNQ, with either the first or second triplet commonly repeated, while the P1.2 family members usually start with HFVQQ and commonly have variations of PQSQ or PKSQ. Grouped within the P1.2 family are four sequences that were previously designated as the P1.33 family. Like sequences in most of the P1.2 family members, these start with HFVQQ and, although they mostly end with SKPTLVP rather than SQPTLVP, they maintain the position of the serine residue.

**Variation within Families**

There was more variation within VR2 (161 unique variants) than in VR1 (73 unique variants). The variation in the VR families was mainly due to changes that could be ascribed to single nonsynonymous base changes. Although

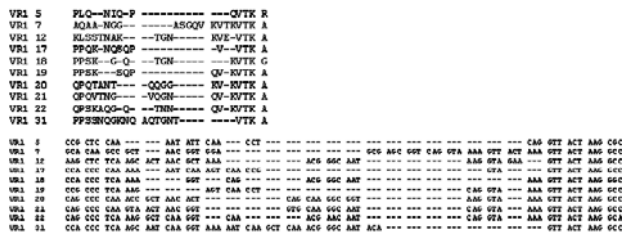


Figure 1. Alignment of the amino acid and corresponding nucleotide sequence of each VR1 family "prototype." For a larger reproduction of this figure, see <http://www.cdc.gov/ncidod/EID/vol10no4/03-0247-G1.htm>

there may be minor differences in the relative contribution of nonsynonymous base changes and insertions or deletions between individual VR families, approximately twice as many variants have arisen as a result of point mutations than from any other type of mutation. The repetition of amino acid motifs or single residues was common within VR2. An example is the repetition of a threonine residue within the VR2 P1.13 family, where there are sequences with three to nine consecutive threonine residues.

**Nomenclature Scheme**

A consultation process was conducted by email among users of the PorA Web site and other interested parties. Several formats for a revised nomenclature were proposed and a request for alternatives made. The consensus opinion was to replace letters with numbers in subtype variant names in the following format: the prefix "P1." followed by the VR1 family name, followed by a dash and then the variant number, followed by a comma and the VR2 variant name in the same format. When a family prototype VR, or first sequence belonging to a family, was identified, no variant number was used; for example, a protein with VR1

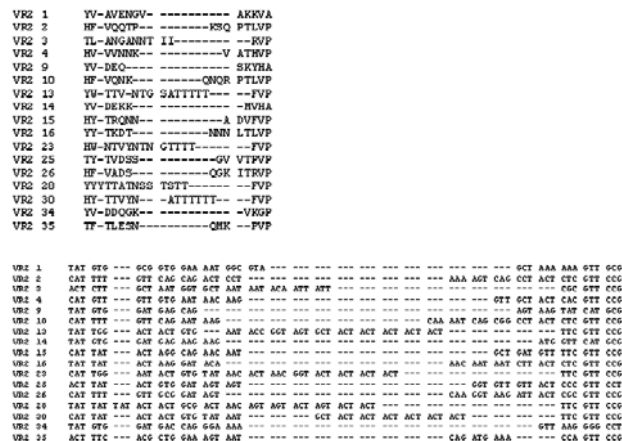


Figure 2. Alignment of the amino acid and corresponding nucleotide sequence of each VR2 family "prototype." For a larger reproduction of this figure, see <http://www.cdc.gov/ncidod/EID/vol10no4/03-0247-G2.htm>

family 5 variant 3, and VR2 family 10 prototype would be written as: P1.5-3,10. This scheme was then used to rename all of the variants examined. The new names of variants are listed in online Appendix Tables 1 and 2, together with the previous nomenclature, peptide sequence, and source or reference. A database accessible through the Internet was established (available from: <http://neisseria.org/nm/typing/pora>).

## Discussion

These analyses confirm that, while diverse, the VR1 and VR2 peptide sequences can be assigned to distinct meningococcal PorA variant sequence families. However, these regions of the PorA protein are likely to be exposed to continual selection imposed by host immune responses, and VR families might evolve over time into different families. The similarity of the P1.2 and P1.10 VR2 families is perhaps a consequence of relatively recent divergence of one VR family into two. Devising a scheme for defining the boundaries of VR families that accurately reflects the evolution of these regions is therefore not possible. Moreover, the high diversity of these sequences presents problems in developing a facile nomenclature. In revising the nomenclature system, we used amino acid sequences, deduced from nucleotide sequences, of the two VRs as the definition of subtype variants. The replacement of letters with numbers in subtype variant designations overcomes the shortage of letters but entails a change of name of variants.

Since MAbs are still routinely used globally for meningococcal serosubtyping, to avoid confusion, family names from the previous nomenclature were retained when possible, and especially when the family prototype was specifically recognized by a typing MAb. The new nomenclature builds on the previous designations but has the advantage of a limitless capacity for expanding the number of variants included. Retaining family names, when they can be shown to be reasonable, results in some minor changes to some family groupings. As meningococci evolve, the use of nucleotide sequencing to determine the VR peptide sequences will be increasingly important for epidemiologic studies and vaccine design, especially as the MAb panel gradually becomes less useful and sequencing technology becomes more available.

In the course of this study, a number of VR sequences that had been deposited previously in GenBank were found, when resequenced, to contain errors and were in fact previously identified variants. These sequences had been given new variant names and, in two cases, were sufficiently novel to warrant the naming of new families. The widespread use and, more importantly, the comparison of VR sequence data among different laboratories require consistency of nomenclature and a high level of data accuracy. One way to achieve this is through a central PorA database

in which sequence electropherograms are submitted for verification before new variant numbers are assigned. We have established a Web site for this role (available from: <http://neisseria.org/nm/typing/pora>). All known variants are listed, and a database query page is provided so that a VR sequence can be typed or pasted in and identified if previously seen. The Web site also includes links to the porB typing and MLST Web pages. The PorA Web site is now in widespread use by the research community and provides a single point of focus to ensure consistency in identifying and naming this important protein.

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Dr. Russell is a scientist whose research interest is the molecular study of infectious disease agents.

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# Antimicrobial Resistance Gene Delivery in Animal Feeds

Karen Lu,\* Rumi Asano,† and Julian Davies\*

Avoparcin, a glycopeptide antimicrobial agent related to vancomycin, has been used extensively as a growth promoter in animal feeds for more than 2 decades, and evidence has shown that such use contributed to the development of vancomycin-resistant enterococci. A cluster that includes three genes, *vanH*, *vanA*, and *vanX*, is required for high-level resistance to glycopeptides. In the vancomycin producer *Amycolatopsis orientalis* C329.2, homologs of these genes are present, suggesting an origin for the cluster. We found substantial bacterial DNA contamination in animal feed-grade avoparcin. Furthermore, nucleotide sequences related to the cluster *vanHAX* are present in this DNA, suggesting that the prolonged use of avoparcin in agriculture led to the uptake of glycopeptide resistance genes by animal commensal bacteria, which were subsequently transferred to humans.

Antimicrobial resistance in bacterial pathogens is a major impediment to successful therapy, and in several instances, bacterial strains have arisen that are refractory to most available antimicrobial treatments (1). Resistance arises by mutation (influencing the target or efflux of the antimicrobial agent) or by the acquisition of resistance genes (encoding antimicrobial or target alteration, or alternate pathways) (2,3). The actual origins of acquired resistance genes are unknown, but environmental microbes, including the strains producing antimicrobial agents, are believed to be important sources (4,5). Substantial genetic and biochemical similarities exist between resistance determinants in antimicrobial agent-producing actinomycetes and resistance genes found in gram-positive and gram-negative pathogens (6–9).

Since vancomycin-resistant enterococci (VRE) were clinically isolated in Europe (1986) and the United States (1987), VRE infections have been reported throughout the world. These infections may be life-threatening because choices for alternative treatment are limited. Concomitant with human use of vancomycin, avoparcin, a closely relat-

ed glycopeptide antimicrobial agent, has been widely used in Europe and other continents as an animal growth promoter (Figure 1). VRE have been isolated, commonly from pigs and chickens fed avoparcin-containing animal feed, and humans coming into contact with the animals (farm

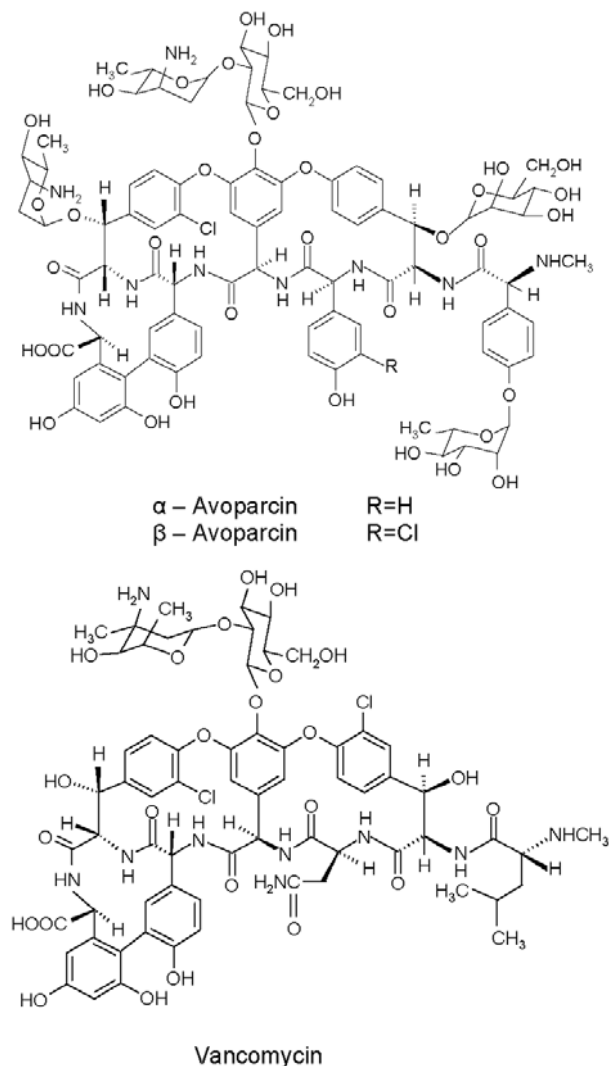


Figure 1. Chemical structures of avoparcin and vancomycin.

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†University of California, Berkeley, California, USA

workers, butchers) have been shown to carry VRE (10–12); identical clones have been found (13). The public health concern about the emergence and dissemination of VRE in food animals and the food supply caused the European Union to ban the use of avoparcin in animal feed in 1997. The discontinued use of avoparcin in animal feed has resulted in a reduction in the number of vancomycin-resistant organisms isolated from animals (14,15).

High-level glycopeptide resistance is conferred by a cluster of three genes, *vanH*, *vanA*, *vanX* (the *van* cluster), plus associated regulatory elements; the cluster is often carried by conjugative transposons (16–18). The *vanH* gene encodes a D-lactate dehydrogenase that provides the requisite D-lactate. *vanX* encodes a highly specific DD-peptidase that cleaves only D-Ala-D-Ala produced endogenously while leaving D-Ala-D-Lac intact. The third gene, *vanA*, encodes an ATP-dependent D-Ala-D-Lac ligase. Replacement of D-Ala-D-Ala by D-Ala-D-Lac in the bacterial cell wall results in a thousandfold reduction in the binding of glycopeptide antimicrobial agents to their peptidoglycan target (19). Studies have demonstrated the presence of *vanHAX* homologs, such as *vanH-ddlN-vanX* (Figure 2), in actinomycete strains producing glycopeptides, and strong structural and functional similarity exists between the various homologs and the *van* cluster of VRE (8,9). Some researchers have proposed that the *vanH*, *vanA*, and *vanX* genes of hospital enterococci may have been acquired en bloc from the actinomycetes (8). Related *vanHAX* gene clusters have been identified in *Paenibacillus* spp. by Patel and coworkers, indicating another possible source of the *van* cluster (20). Regardless of the microbial source, the feeding of crude antimicrobial preparations to animals is plausible as a delivery process for transferring the cognate antimicrobial resistance genes between producing strains and the commensal bacteria of animals (21); the concomitant selection for resistance would ensure the survival of rare resistant strains. We provide evidence that a DNA-encoding homolog of the *van* cluster is a contaminant of feed-grade avoparcin and propose that animal use both created and selected for glycopeptide-resistant strains. The emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA) is a recent sequela to this train of events involving the *van* gene clusters (22).

## Materials and Methods

### DNA Extraction from Avoparcin

A suspension (0.7 mL) of avoparcin (Roche, Sydney, Australia) in H<sub>2</sub>O (100 mg/mL) was centrifuged in a 1.5-mL Eppendorf tube for 6 min and the supernatant, after being shaken with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), was centrifuged at 16,000  $\times$  g for 3 min. The aqueous phase was subjected to two additional phe-

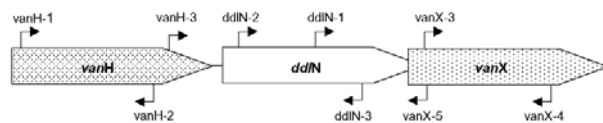


Figure 2. The *vanHAX* cluster of *Amycolatopsis coloradensis* NRRL 3218.

nol-chloroform extractions. The nucleic acid in the pooled aqueous fractions was precipitated with ethanol; the pellet was recovered by centrifugation and further purified by using a GeneClean spin kit (BIO 101 Systems, Carlsbad, CA) and resuspended in 100  $\mu$ L of double-distilled H<sub>2</sub>O. The DNA concentration was measured with a fluorometer (Model TKO100, Hoefer Scientific Instruments, San Francisco, CA).

### PCR Amplification of 16S rDNA Sequences

Primers 16S 440F and 16S 1491R (Table) were designed to amplify partial 16S rDNA sequences. The polymerase chain reaction ([PCR] contained 2 mmol/L MgCl<sub>2</sub>, 0.16 mmol/L dNTP, 0.4  $\mu$ mol/L of each primer, *Taq* polymerase (1 U), 3–15 ng template, and 5% dimethyl sulfoxide (DMSO). PCR was done in a MiniCycler (MJ Research, Waltham, MA) by using the following program: 96°C, 3 min; 96°C, 30 s; 60°C, 45 s; 72°C, 1 min 30 s; 35 cycles; and 72°C, 10 min.

### PCR Amplification of *vanH*, *ddlN*, and *vanX* Sequences

Different combinations of PCR primers (9) were used to amplify the entire *van* cluster (Table). Reaction conditions were as described previously.

### Cloning of *vanH*, *ddlN*, *vanX*, and Partial 16S rDNA Genes

PCR products were cloned by using vector pCR 2.1-TOPO (Invitrogen, Burling, Ontario, Canada) according to the manufacturer's instructions, and the insertion size was

Table. Primer sequences for genes *vanH*, *ddlN*, *vanX* and partial 16S rDNA

Primer	Primer sequences
vanH-1	5'-CAC ATC GA(C/T) GTG GAA TAC GC-3'
vanH-2	5'-CAG TCG GCG TAG AAG ATG CC-3'
vanH-3	5'-GAG GAA GGC ATC TTC TAC GC-3'
ddlN-1	5'-ACG (G/C)CA GTA CGA C(G/T)C GAA G-3'
ddlN-2	5'-T(G/T)C CTG GA(A/T) GCT (G/C)TG CGA C-3'
ddlN-3	5'-G(A/G)T AAC GGC TGT ACG AGG TC-3'
vanX-3	5'-CCA CGT GGG ACA ACT TCA C-3'
vanX-4	5'-CAG (C/G)(G/T)T GTA GTG CCA CCA CTC-3'
vanX-5	5'-TCA CCA GAT ATC CGT CCA CC-3'
16S 440F	5'-AGC AGG GAA GAA GCG (A/T/C)(A/G)A GT-3'
16S 1491R	5'-CGG CTA CCT TGT TAC GAC TTC-3'

confirmed by a second PCR. Plasmid DNA was extracted by using the Concert rapid plasmid miniprep system (Invitrogen).

### DNA Sequence Analysis

Cycle sequence reactions were carried out with a BigDye terminator DNA sequencing kit (Applied Biosystems, Foster City, CA) with plasmid DNA templates. The cycle sequence program was as follows: 96°C, 1 min; 96°C, 30 s; 50–60°C (dependent on different primers and fragments), 15 s; 60°C, 4 min, for 25 cycles. Excess oligos and dyes were removed by using CentriSep spin columns (Princeton Separations, Aldelphia, NJ). Reaction products were sequenced by the Nucleic Acid and Protein Service, University of British Columbia, using an ABI PRISM 377 sequencer. Sequences were analyzed by using the standard nucleotide-nucleotide BLAST program (National Center for Biotechnology Information, Bethesda, MD), and comparisons were carried out by using CLUSTAL W (European Bioinformatics Institute, Cambridge, UK).

### Results and Discussion

Direct extraction of avoparcin powder with phenol/chloroform/isoamyl alcohol provided substantial amounts of DNA (30.5 µg/g of avoparcin) (Figure 3A). PCR amplification of the DNA with oligonucleotide primers specific for a region of streptomycete 16S rRNA gave a single amplicon (Figure 3B), which was sequenced and shown to be 16S closely related to that of *Amycolatopsis coloradensis*, the producer of avoparcin. Figure 3B shows similarities between the 16S rRNA of species that produce glycopeptide antimicrobial agents.

To examine for the presence of genes involved in glycopeptide resistance from the antimicrobial agent-derived DNA, we used the DNA primers described by Marshall et al. (9). The amplicons (Figure 4) were cloned, sequenced, and assembled, indicating a *van*-like cluster closely related to that found in *A. orientalis* and *Streptomyces toyocaensis*. Control reactions run without added template were negative.

The genes encoded three putative proteins showing >50% amino acid identity to the Van H, A, and X proteins of VRE (Figure 2). All of the clusters have translational overlaps between the *vanA* and *vanX* genes and their homologs, suggesting cotranslational regulation of expression. This finding clearly implies that the *van* cluster must be transferred and acquired in toto from any source organism.

We suggest that the use of crude avoparcin preparations in animal feeds from 1975 to 1996 was the origin of the *vanHAX* cluster in the genesis of VRE (and possibly that found in VRSA) (22,23). Large amounts of avoparcin were

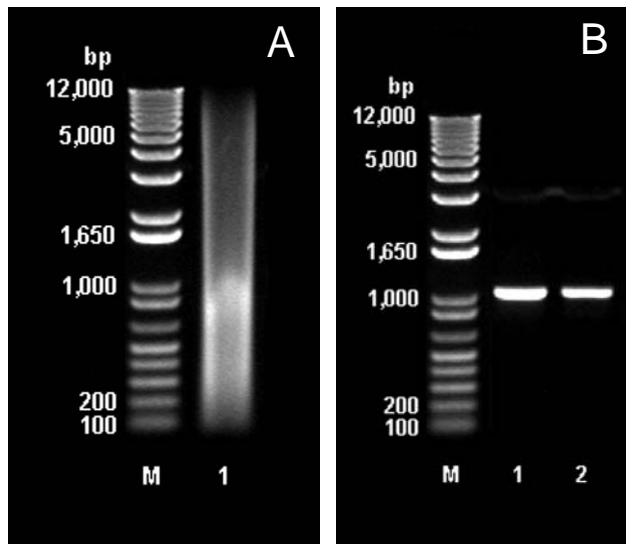


Figure 3. A, Molecular mass of total DNA extracted from animal feed-grade avoparcin. M: 1 kb plus DNA ladder (Invitrogen, Burlington, Ontario, Canada). Lane 1: sample of total DNA extracted from animal feed-grade avoparcin. B, Polymerase chain reaction amplification of partial 16S rDNA (1051 bp) with primers 16S 440F and 16S 1491R. M: 1 kb plus DNA ladder. Lane 1: DNA extracted from animal feed-grade avoparcin. Lane 2: DNA of the avoparcin producer *Amycolatopsis coloradensis* NRRL 3218.

used in animal feed; in Denmark, for example, total vancomycin use in 1994 amounted to 24 kg, whereas avoparcin use in animals was 24,000 kg (24). During their entire lives, broiler chickens received 15 mg/kg and pigs 20–40 mg/kg of antimicrobial agent in their feed. Each pig was fed 5–10 g of the crude drug for its life span and, consequently, received a steady dose of DNA encoding vancomycin resistance. In Europe, an estimated 100 mg of antimicrobial agents are used in animal feed for the production of 1 kg of meat for human consumption. We believe that this regimen would have favored the selection and maintenance of rare bacterial transformants carrying the resistance genes. If one bears in mind that large numbers of pigs and chickens were exposed to the antimicrobial agent, the probability of gene pick-up by bacterial commensals in the animal gastrointestinal tract would be favored, and once incorporated into a gut commensal genome, further dissemination would have followed under antimicrobial selection. The finding that organization of the *van* cluster in contaminating DNA of the feed is identical to that in VRE, with overlapping reading frames typical of translational coupling of gene expression between the *vanA* and *vanX* homologs (9), reinforces this supposition.

The mechanism by which a *van* cluster becomes functionally integrated into bacteria is not known. We propose that intestinal bacteria were the original recipients of the DNA; many of the resident strains are known to be competent for DNA uptake (25,26). However, mere uptake is

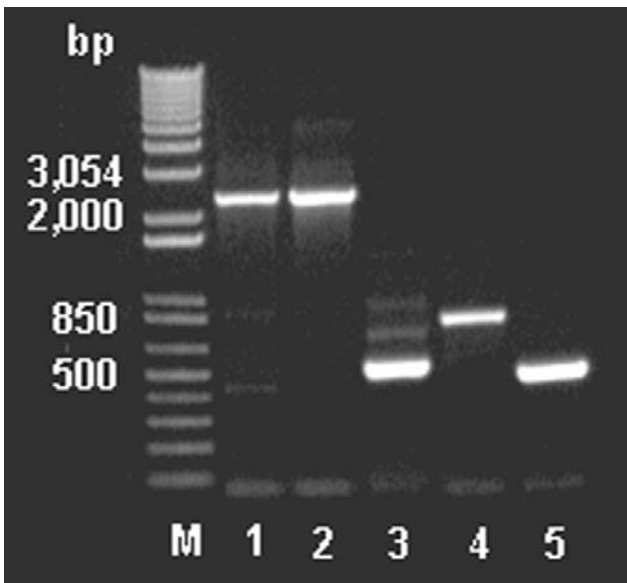


Figure 4. Polymerase chain reaction amplification of the partial *vanHAX* clusters from DNA extracted from animal feed-grade avoparcin and the antibiotic producer *Amycolatopsis coloradensis* NRRL 3218, and genes *vanH*, *ddIN*, and *vanX* from DNA extracted from animal feed grade avoparcin. M: 1 kb plus DNA ladder. Lanes 1-2: the partial *van* cluster (2.3-2.4 kb) amplified with primers *vanH*-1 and *vanX*-4; lane 1: DNA extracted from animal feed grade avoparcin; lane 2: DNA of the avoparcin producer *A. coloradensis* NRRL 3218. Lane 3: *vanH* (500 bp) amplified with primers *vanH*-1 and *vanH*-2. Lane 4: *ddIN* (850 bp) amplified with primers *ddIN*-2 and *ddIN*-3. Lane 5: *vanX* (500 bp) amplified with primers *vanX*-3 and *vanX*-4.

not sufficient for function, and the actinomycete genes differ from VRE genes in their G+C content (approximately 65% vs. 50%) and codon usage. Given the enormous complexity of bacterial populations in the mammalian gastrointestinal tract (27,28), we assume that a variety of intestinal species may have incorporated the resistance-encoding DNA; expression (at low levels) would have been rare, depending on the compatibility of the *van* genes with the transcription and translation system of the host. Under constant antimicrobial selection pressure, translationally competent sequences would have developed by mutation; this would not necessarily have occurred in enterococci. Nonetheless, the conversion (evolution) of the actinomycete genes into functional enterococcal genes likely would have required many generations of growth under constant selection, and any intermediate stages in this process are a matter of speculation; however, once established on conjugative transposons, the genes would be readily disseminated (17,29). A number of similar *van* clusters have been identified in different bacterial species, and whether these evolved independently or by divergent evolution is unknown.

The finding of resistance genes in crude antimicrobial products intended to be fed to animals adds to the already strongly voiced opinion that use of antimicrobial agents in this way constitutes a serious public health concern and further emphasizes the need for prohibiting the use in animal feed of all antimicrobial agents that are employed in human therapy. This ban should include structurally or biologically related antimicrobial agents and the use of any compound with the potential to select for cross-resistance to another antimicrobial agent (15,30). The use of avoparcin in Denmark was prohibited in 1995 and in the European Union in 1997. Subsequently, several other antimicrobial growth promoters were banned (31,32). However, the United States and Canada permit the use of many such products, including penicillin, tetracycline, macrolides, and sulfonamides. Nonhuman applications of antimicrobial agents, such as in agriculture and aquaculture, should employ only chemically and biologically distinct classes of compounds developed specifically for that purpose. Clearly such measures should be combined with a requirement for rational and prudent measures for antimicrobial use in the human population.

Many antimicrobial agents (or their close structural relatives) have been used extensively as animal-feed additives. In almost all cases, crude antimicrobial preparations are used, and thus the antimicrobial agent acts as a carrier for its cognate resistance genes. These delivery systems provide the opportunity for resistant strains of bacteria to evolve and so create an enormous gene pool for antimicrobial resistance determinants in the environment.

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Ms. Lu is the senior technician and administrator of the Davies Laboratory, Department of Microbiology and Immunology, University of British Columbia. Her principal interests are the characterization of antimicrobial agent-resistant bacteria and their mechanisms of resistance. She is currently working on a reporter system to classify antimicrobial activity.

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# Flea-borne *Bartonella grahamii* and *Bartonella taylorii* in Bank Voles

Kevin J. Bown,\* Malcolm Bennett,\* and Michael Begon\*

*Bartonella* species are increasingly associated with a range of human and animal diseases. Despite this, we have a poor understanding of the ecology and epidemiology of many species, especially those circulating in wild rodent populations. Previous studies have demonstrated that a diverse range of *Bartonella* species are abundant in wild rodent populations; little is known regarding their modes of transmission, although both direct and indirect routes have been suggested. In this study, with bank voles (*Clethrionomys glareolus*) as the host species, we demonstrate that the rodent flea *Ctenophthalmus nobilis* is a competent vector of at least two *Bartonella* species, *B. grahamii*, which has previously been associated with human infection, and *B. taylorii*. In contrast, no evidence of either horizontal or vertical transmission was seen in bank voles inoculated with *B. taylorii* maintained in an arthropod-free environment; this finding suggests that fleas may be essential for transmitting some *Bartonella* species.

The genus *Bartonella* currently contains 19 species of gram-negative bacteria that parasitize the erythrocytes of vertebrate hosts, and an increasing number of species are now considered as emerging infections of medical and veterinary importance (1). In addition to humans and domesticated animals, they have also been isolated from a variety of wild mammal species, including cervids, ruminants, carnivores, and rodents (1–3). Of these, rodents are perhaps the best studied, with high prevalences of *Bartonella* infections, coupled with highly diverse species and strains (4–7). Human infections with *Bartonella* species of rodent origin have been reported from both sides of the Atlantic: in the United States, *B. elizabethae*, associated with endocarditis, *B. washoensis*, associated with cardiac disease, and *B. vinsonii* subsp. *arupensis*, causing fever and neurologic symptoms (8–10); in Europe, *B. grahamii*, isolated from the eye of a patient with neuroretinitis (11).

While the association of *Bartonella* of rodent origin with human disease continues to increase, our understanding of the ecology and epidemiology of these infections is

scant. Fundamental to this endeavor would be clarifying their mode(s) of transmission. *Bartonella* species are generally considered to be transmitted by arthropod vectors, and *Bartonella* DNA has been detected in fleas and ticks collected from both wild and domestic animals (12–16). However such findings do not necessarily prove vector competence, and vectors have only been conclusively identified for a few species: the sand fly (*Lutzomyia verrucarum*) for *B. bacilliformis* (17), the body louse (*Pediculus humanus*) for *B. quintana* (18), and the cat flea (*Ctenocephalides felis*) for *B. henselae* (19). Anecdotal evidence exists for the role of ticks as vectors of at least some *Bartonellae* (20–22). For rodent *Bartonellae*, two vectors have been suggested. The oriental rat flea (*Xenopsylla cheopis*) was demonstrated to be a competent vector of an unidentified *Bartonella* species that infected bank voles (*Clethrionomys glareolus*) (23), and the vole ear mite (*Trombicula microti*) was proposed as a vector of *B. vinsonii* (24). However, no experimental transmission studies have been undertaken in which the *Bartonella* species involved could be accurately identified by, for example, using a molecular approach. In addition, vertical transmission has been suggested as a potential mechanism by which infection may be maintained within a population (25), and experimental data suggest that transplacental transmission occurred in BALB/c mice infected with *B. birtlesii*, although no viable fetuses were bacteremic (26).

The aim of this study was to determine the potential for fleas, collected from a population of bank voles in which *Bartonella* infections were known to be endemic, to transmit infection to naïve bank voles. In addition, the potential importance of direct horizontal or vertical transmission was investigated.

## Materials and Methods.

Twenty fleas were collected from six bank voles sampled in a mixed woodland in northwest England (53°20.6N, 3°02.4W) where previous studies had shown the prevalence of *Bartonella* infection in bank voles was approximately 60% (5). These fleas were added to a rodent “arena,” measuring 1.2 m x 1.2 m in a temperature-

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controlled room. The arena contained sawdust as substrate, hay and shredded paper as bedding, and Longworth traps (Abingdon, UK) set on prebait as nest boxes. No *Bartonella* spp. had been used in experiments in the arena before the introduction of the fleas, and no fleas had previously been kept in the arena. The arena had been kept free of bank voles for 2 weeks before this study began. Twenty-eight captive-bred bank voles from a *Bartonella*-free colony maintained at the University of Liverpool were added to the arena immediately after the fleas were introduced. All of these voles had tested negative for *Bartonella* infection before entering the arena.

Four weeks after the bank voles were added to the arena, all were euthanized, and blood samples were collected by cardiac puncture. In addition to the 28 voles originally introduced to the arena, one female had produced a litter, and the two pups produced were also humanely killed and had sterile blood samples collected. Fleas were collected from all rodents and kept in individual tubes (1 per rodent) containing 70% ethanol. A sample of fleas from bedding within the arena was collected at the same time. All fleas were identified to species level (27).

Isolation of *Bartonella* spp. from the blood samples was undertaken by plating freeze-thawed blood onto Colombia blood agar plates enriched with 5% horse blood. Plates were incubated at 37°C and 5% CO<sub>2</sub> for up to 14 days. Isolates putatively resembling *Bartonella* spp. colonies were further characterized by polymerase chain reaction (PCR). Individual colonies were prepared by boiling in 100 µL of sterile deionized distilled water for 10 min. Five microliters of this preparation were used as template. Each 50-µL reaction contained 1.25 U of Taq polymerase, 200 mmol of each dNTP, 1.5 mmol of MgCl<sub>2</sub>, and 30 pmol of each primer. Initial characterization used primers QHVE1 and QHVE3 (28) that target the 16S–23S rRNA intergenic spacer region. PCR products from positive samples were purified by using the Promega Wizard PCR Preps kit (Promega, Madison, WI), and then digested with *Hae*III as previously described (29). Samples relating to each REA pattern were then analyzed by using primers BhCS781.p and BhCS1137.n (30), which target the citrate synthase (*gltA*) gene. After purification these PCR products were sequenced with an ABI 377 automated sequencer, and the sequences were compared with previously published sequences by using the BLAST program from the National Center for Biotechnology Information Web site (available from: <http://www.ncbi.nlm.nih.gov/BLAST/>).

Horizontal and vertical transmission experiments were undertaken using 16 bank voles, housed, in the absence of fleas, in cages containing male-female pairs approximately 4 weeks of age, one or both of which was injected through the footpad with approximately 10<sup>6</sup> CFU of

*Bartonella taylorii*. In two of the cages both voles were inoculated, in two only the male vole was inoculated, and in four others only the female vole was inoculated. Pairs were kept until they had produced a litter. Blood samples were taken at day 0, when the voles were inoculated, at day 10 to confirm infection status of the adults, and 8 weeks later when litters were between 7 and 14 days old. Isolation attempts were carried out as described.

## Results

Twenty-one of the 28 blood samples from the bank voles produced colonies resembling *Bartonella* spp., and all of these were confirmed as *Bartonella* spp. by PCR. Restriction enzyme analysis of the resulting PCR products showed that two different *Bartonella* genotypes were present in the bank voles (Figure). Sequence analysis of the *gltA* gene showed these to represent *B. taylorii* (16 isolates) and *B. grahamii* (6 isolates) (one bank vole was coinfecting with both). In addition to the original 28 voles added to the arena, two pups were sampled that had been suckling from a bacteremic female. Neither was bacteremic.

A total of 217 fleas were collected from the 28 bank voles (mean 7.75 fleas per vole). Only one species of flea was identified, *Ctenophthalmus nobilis nobilis*. Ten pools of five randomly selected fleas collected from the voles were tested for *Bartonella* spp. DNA using the *gltA* PCR. All pools tested positive, and of 10 individual fleas collected directly from the arena itself, 7 tested positive for *Bartonella* spp. DNA. Four were positive for *B. taylorii*, one for *B. grahamii*, and two for both.

None of the naïve adults involved in the horizontal transmission experiment acquired infection directly from

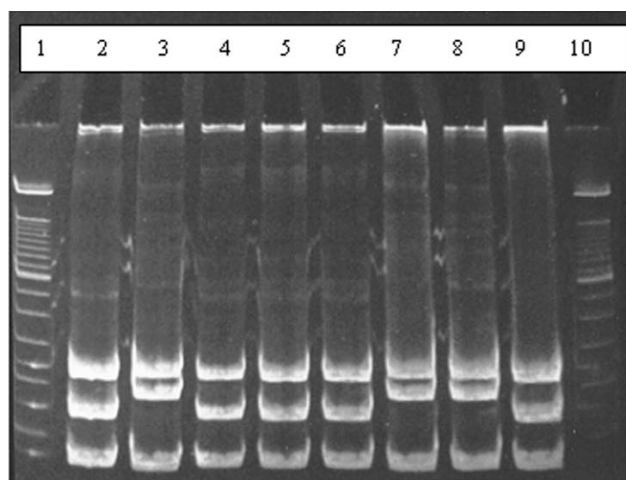


Figure. Results of restriction enzyme analysis on 7% polyacrylamide gel showing the two restriction patterns obtained by digesting polymerase chain reaction products with *Hae*III. Lanes 2, 4, 5, 6, and 9 show digestion of amplicons of *Bartonella taylorii*; lanes 3, 7, and 8 show digestion of *B. grahamii* amplicons; lanes 1 and 10 contain molecular weight markers.



its mate, despite that all inoculated animals remained bacteremic throughout the experiment. Seven of the eight pairs of voles produced a litter, one of the pairs in which the female alone was inoculated did not. A total of 20 young were produced from the seven litters, with litter sizes ranging between one and five offspring (mean 2.86 offspring per litter). No bacteremia could be detected in any of the offspring, whether only one or both parents had been inoculated.

## Discussion

This study shows that fleas are efficient vectors of at least some rodent bartonellae. Twenty one of 28 (75%) naïve bank voles housed with wild-caught fleas for 4 weeks became bacteremic, 16 voles (57.1%) infected with *B. taylorii* and 6 voles (21.4%) infected with *B. grahamii*. Similarly, each of 10 pools of 5 fleas collected showed the presence of *Bartonella* spp. DNA within them, and 7 of 10 individual fleas were also positive. Fleas have previously been implicated in the transmission of *B. henselae* infections of cats (19,31,32), and *Bartonella* DNA has previously been detected in fleas collected from rodents (14,15), but no recent experimental studies on the role of fleas in the transmission of rodent bartonellae have been reported since early studies by Krampitz (23) indicated that fleas could transmit an unidentified *Bartonella* species. In fact, two different *Bartonella* species could be transmitted by a single species of flea, suggesting little vector-bacteria specificity.

On the other hand, no transmission occurred between infected and susceptible animals when housed together in the same cage in the absence of fleas, and no transmission could be detected from parent to offspring, although larger numbers of animals may be needed to confirm that such transmission does not occur. This absence of vertical transmission agrees with results of a study of cats infected with *B. henselae* (33,34), but Kosoy and colleagues (25) found that *Bartonella* could be isolated from the neonates and embryos of naturally infected North American rodents, while transplacental transmission of *B. birtlesii* was also reported in BALB/c mice, although none of the viable offspring in that study were bacteremic (26).

These findings are of potential public health importance: *B. grahamii* has previously been associated with human disease (11), although the pathogenic potential of *B. taylorii* is as yet unknown. Furthermore, the implication of fleas in the transmission of these rodent *Bartonella*, as well as *B. henselae* (19,31) suggests that fleas may be involved in the transmission of many other rodent *Bartonella* species, some of which have already been shown to be pathogenic to humans. Whether the route of rodent to human transmission is likely to be due to flea transmission or through direct contact, such as bites or

scratches as is commonly the case for *B. henselae* (1), needs to be investigated.

The exact route by which fleas transmitted *Bartonella* to susceptible rodents remains unclear. Future work should seek to distinguish the role of fecal contamination and then the role of scratching (32) from direct transmission through feeding. Investigating the efficiency of different flea species in transmitting a variety of *Bartonella* species would be valuable as would determining whether fleas infected with a number of *Bartonella* species transmit one species more efficiently than the others. Studies such as these would help expand the current knowledge on vector-*Bartonella* specificity and determine its importance in influencing the diversity of *Bartonella* species.

Dr. Bown is currently a research associate in the Faculty of Veterinary Science at the University of Liverpool. His interests focus on the ecology of wildlife diseases, particularly those caused by vector-borne bacteria.

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# Restaurant Inspection Scores and Foodborne Disease

Timothy F. Jones,\* Boris I. Pavlin,† Bonnie J. LaFleur,† L. Amanda Ingram,\* and William Schaffner†

Restaurants in the United States are regularly inspected by health departments, but few data exist regarding the effect of restaurant inspections on food safety. We examined statewide inspection records from January 1993 through April 2000. Data were available from 167,574 restaurant inspections. From 1993 to 2000, mean scores rose steadily from 80.2 to 83.8. Mean inspection scores of individual inspectors were 69–92. None of the 12 most commonly cited violations were critical food safety hazards. Establishments scoring <60 had a mean improvement of 16 points on subsequent inspections. Mean scores of restaurants experiencing foodborne disease outbreaks did not differ from restaurants with no reported outbreaks. A variety of factors influence the uniformity of restaurant inspections. The restaurant inspection system should be examined to identify ways to ensure food safety.

More than 54 billion meals are served at 844,000 commercial food establishments in the United States each year (1); 46% of the money Americans spend on food goes for restaurant meals (2). On a typical day, 44% of adults in the United States eat at a restaurant (1). Of a mean 550 foodborne disease outbreaks reported to the Centers for Disease Control and Prevention each year from 1993 through 1997, >40% were attributed to commercial food establishments (3). Preventing restaurant-associated foodborne disease outbreaks is an important task of public health departments.

Restaurants in the United States are regularly inspected by local, county, or state health department personnel. The guidelines of the U.S. Food and Drug Administration state that “a principal goal to be achieved by a food establishment inspection is to prevent foodborne disease” (4). Although restaurant inspections are one of a number of measures intended to enhance food safety, they are a highly visible responsibility of local health departments. In many parts of the country, restaurant inspection scores are easily accessible to the public through the Internet or are

disseminated through local news media. We postulated that an inspection system that effectively addressed the goal of improving food safety would be uniform, consistent, and focused on identifying characteristics known to affect food safety. We examined data on restaurant inspections in the state of Tennessee to determine whether the system there demonstrated such characteristics.

## Methods

Statewide restaurant inspection data from Tennessee from January 1993 through April 2000 were analyzed. Semiannual inspections were required of all restaurants with permits for preparing and serving food; all routine inspections during this period were included in the analysis. Special inspections performed in response to customer complaints or to follow-up on deficiencies noted in semiannual inspections were not included. We did not include inspections of schools, correctional facilities, and bars that did not serve food. Inspections were performed by state health department employees, or by county health department employees in most metropolitan areas of the state, in accordance with uniform state laws and regulations. All inspectors undergo uniform training and certification by state health department management personnel. To avoid skewing results by including persons performing very few inspections per year, when comparing mean inspection scores by inspector, we included those performing at least 100 inspections during the study period.

Inspections were performed by using standardized forms including 44 scored items with a possible total score of 100. Of those 44 items, 13 were designated as “critical” (Appendix). Critical items are violations “which are more likely to contribute to food contamination, illness, or environmental degradation and represent substantial public health hazards and [are] most closely associated with potential foodborne disease transmission” (4). Data available for each inspection included overall score, specific violations cited, establishment name and identification number, county, date of inspection, inspector, and time spent on inspection.

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For comparison purposes, a convenience sample of 19,700 inspections of 2,379 restaurants known to serve distinct types of international or regional cuisine were analyzed. In addition, a convenience sample of 46,700 inspections of 5,300 restaurants were compared on the basis of type of table service. These restaurants were selected based on being well-known to investigators as to type of service or cuisine. Fast-food restaurants were defined as establishments where food was paid for before eating. Full-service restaurants were defined as establishments where patrons were served at tables and meals were paid for after consumption. Establishments that were difficult to classify or not known to investigators were not included. Data were entered in a centrally maintained database and were analyzed with Excel (Microsoft, Redmond, WA), SAS 8.0 (SAS, Cary, NC), and EpiInfo 6.2 software (5).

**Results**

All commercial establishments preparing or serving food in Tennessee are required to hold a permit from the Tennessee Department of Health. Tennessee has approximately 13,000 restaurants licensed and approximately 145 restaurant inspectors. Data were available from 167,574 restaurant inspections, involving 29,008 unique restaurants and 248 inspectors during the study period. During this period, individual restaurant scores were 13–100; the mean was 82.2, and the median was 83 (Figure 1). Among 190 inspectors performing at least 100 inspections during the study period, mean inspection scores of individual inspectors were 69–92, with a median of 82 (Figure 2). Mean scores of restaurants within each of the 95 counties in Tennessee were 75–88. From 1993 to 2000, the mean inspection score rose steadily from 80.2 to 83.8, and the mean number of violations cited per inspection fell from 11.1 to 9.9.

During routine restaurant inspections, the most commonly cited violations were for unclean surfaces of equipment that did not contact food and floors or walls appearing unclean, poorly constructed, or in poor repair (Table). None of the 12 most commonly cited violations were among those designated as “critical” food safety hazards. The critical violation most commonly cited was the improper storage or use of toxic items (for example, storing cleaning fluids on a shelf next to food), which was the 13th most commonly cited violation during routine inspections.

Among restaurant inspections with a total score of >80, at least one critical violation was cited in 44% of those inspections (mean number of critical violations was 0.6, mean number of noncritical violations was 6.3). A critical violation was cited in 9,127 inspections with a final score >90. Among inspections with scores of 60 to 80, a mean of 2.4 critical and 11.4 noncritical violations were cited; for

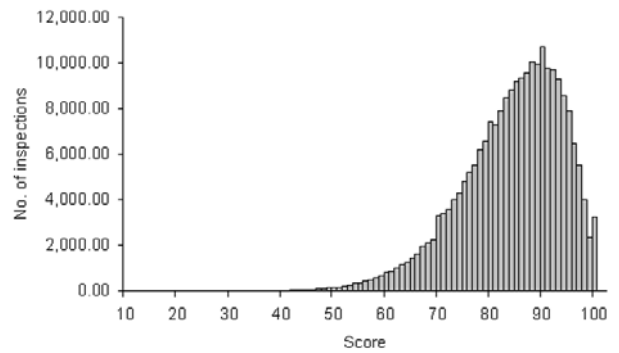


Figure 1. Distribution of scores of restaurants inspected statewide from July 1993 to June 2000, based on a standardized inspection with 44 scored items and a maximum score of 100.

inspections with a score <60, the means were 5.4 and 16, respectively. In 1,698 inspections with a score of 60 to 80, no critical violations were cited.

During this period, restaurants with a score >60 tended to have fairly stable scores on subsequent inspections, with a mean drop of 2 points on the subsequent inspection (Figure 3). Establishments scoring <60 had a mean improvement of 16 points on the subsequent routine inspection, with an additional mean increase of 5 on the next inspection.

Restaurant inspection data were available from 49 restaurants that were identified as the source of foodborne disease outbreaks investigated by health departments in Tennessee from 1999 to 2002. The mean score of the last routine inspection before the reported outbreak was 81.2, and the mean score of the inspection previous to the most recent inspection was 81.6. These scores do not differ significantly from the mean scores of all restaurant inspections during the study period. The rank order of most commonly cited critical violations on routine inspections of restaurants subsequently involved in outbreaks was similar to restaurants not involved in outbreaks. While the two most common critical violations (proper storage of toxic items and good handwashing and hygienic practices) were more likely to have been cited during the two routine

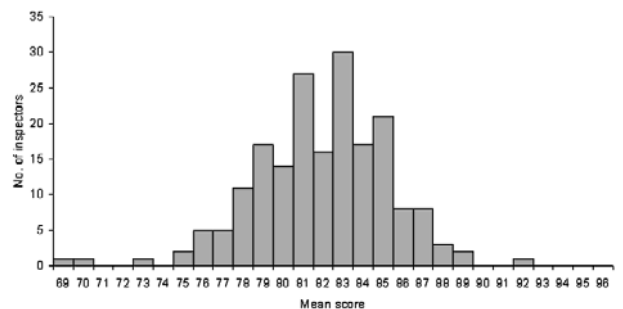


Figure 2. Mean score of all restaurants inspected by each inspector, for inspectors performing at least 100 inspections during the study period.

## RESEARCH

Table. Number of times each of the 15 most common violations were cited on routine restaurant inspections statewide, 1993–2000

Standard in violation	Frequency
Nonfood contact surfaces of equipment and utensils clean	142,924
Floors constructed, drained, clean, good repair, covering, installation, dustless cleaning methods	142,812
Walls, ceilings, attached equipment, constructed, good repair, clean surfaces, dustless cleaning methods	136,178
Food-contact surfaces of equipment and utensils clean, free of abrasives, detergents	127,156
Non-food contact surfaces designed, constructed, maintained, installed, located	111,813
Food protection during storage, preparation, display, service, transportation	101,126
Food (ice) contact surfaces designed, constructed, maintained, installed	96,657
Premises maintained free of litter, unnecessary articles, cleaning maintenance equipment properly stored	91,422
Toilet rooms enclosed, self-closing doors, fixtures good repair, clean, hand-cleanser, sanitary towels, hand-drying devices provided, proper waste receptacles	88,140
Single-service articles, storage, dispensing	81,562
Containers or receptacles, covered, adequate number, insect and rodent proof, frequency, clean	78,143
Lighting provided as required, fixtures shielded	71,453
Toxics items properly stored, labeled, used <sup>a</sup>	70,995
Thermometers provided and conspicuous	69,595
Food protection during storage, preparation, display, service, transportation	69,059

<sup>a</sup>“Critical” item.

inspections before an outbreak occurred at a restaurant, the number of reported outbreaks is small, and the conclusions that can be drawn from this observation are limited.

Under state law, restaurants in Tennessee are inspected once every 6 months. The median time between successive inspections during this period was 175 days; 88% of inspections were performed from 90 to 270 days after the previous inspection. Mean scores were similar in restaurants inspected less than or more than 180 days since the previous inspection (81.7 and 82.7, respectively) and in restaurants inspected within 200 days compared to >270 days since the previous inspection (81.9 and 83.7, respectively).

Fast-food restaurants (mean score = 79.9) had mean scores similar to independent (80.9) or chain (82.1) full-service restaurants. Small variations were noted in mean scores of restaurants serving specific types of cuisine, such as Thai (83.1), barbeque (82.9), pizza (82.3), Italian (81.0), Chinese (77.7), Mexican (77.4), Japanese (76.4), and Indian (74.8) foods.

### Discussion

These data demonstrate that, during a 7-year period in Tennessee, routine restaurant inspection scores varied substantially over time, by region, and by person performing the inspection. While regional variations in the general quality of food service establishments are possible, this factor is unlikely to account for a substantial proportion of the observed differences. Restaurant inspections performed by a single observer are difficult to standardize and easily influenced by subjective interpretation. Further analyses can be performed that examine the variation in scores on the basis of such things as demographic characteristics of inspectors and time since last standardized training; these analyses can also be done prospective studies of interobserver variability at the same establishments.

All restaurant inspections in Tennessee during this period were performed under the same laws and procedures and using standard data collection forms. New inspectors undergo standardized training before performing inspections alone, though during this study period no mechanism for formal periodic restandardization after initial training existed. Since this study period (and independently of this study) the health department has instituted statewide retraining of all inspectors, regardless of length of experience. Whether periodic standardized retraining affects the variables assessed in this study is yet to be determined.

Despite the ubiquity of restaurant inspections, few studies have been published about the correlations between restaurant inspection scores or violations and foodborne illness, and the conclusions are conflicting (6–12). Methodologic problems, including the rarity of reported foodborne outbreaks in relation to the number of restaurants and the small percentage of suspected foodborne illnesses linked to epidemiologically confirmed, restaurant-associated outbreaks, make such analyses difficult. The intensity of surveillance for foodborne disease can markedly influence the number of foodborne disease outbreaks reported in a jurisdiction, and a substantial proportion of restaurant-associated foodborne illnesses probably goes unreported. This study did not assess foodborne illness as an endpoint but rather examined characteristics of an inspection system that would be expected to be associated with a consistent, predictable, and reliable foodborne illness prevention system. The limited data available on outbreaks in Tennessee suggest that restaurant inspection scores alone do not predict the likelihood of a foodborne outbreak occurring in a particular establishment.

We are not aware of published data addressing which items on a routine restaurant inspection are demonstrated to lead to improved food safety within an establishment.



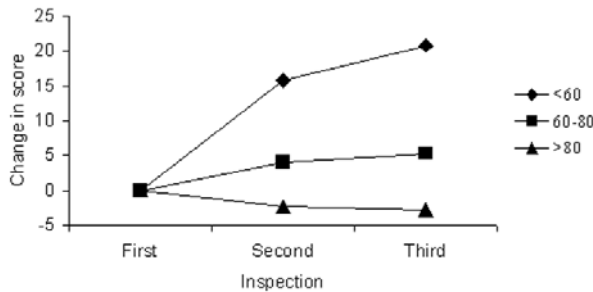


Figure 3. Mean change in scores in subsequent two inspections, for restaurants with an initial score on routine inspection of <60, 60–80, or >80.

The Tennessee Department of Health inspection protocol and the federal Food Code (4) after which it is modeled include assessment of a variety of factors of limited importance in directly preventing foodborne illness. These items include condition surfaces that do not contact food, floors, walls and ceilings, lighting, and ventilation. Such factors would be expected to substantially influence an observer's impression of overall cleanliness and safety of an operation, but isolated characteristics have not been shown to correlate with food safety. A substantial number of inspections with a final score of >90 also had critical violations; likewise, some restaurants with scores <80 had no critical violations. While most common violations are noncritical items, these data serve as a reminder that overall score alone is not necessarily a sufficient measure of restaurant safety. A number of studies have examined the effect of inspection frequency on restaurant sanitation (9,13–16). We did not observe a meaningful difference in scores on the basis of time since previous inspection, although because of state laws requiring inspections every 6 months, the variation in intervals was limited. Data from other programs with more variation in inspection frequency might be helpful in assessing the potential effect of time since last inspection.

Restaurant inspections serve an additional goal of ensuring immediate physical safety of patrons and workers in the environment. Further studies to determine the most efficient and effective methods for assessing factors associated with food safety will be important to help improve the inspection system. Recent introduction of Hazard Analysis and Critical Control Points systems in many areas of the foodservice industry are an attempt to focus proactively on issues important to food safety (4).

Given the universal performance of restaurant inspections in the United States, no large group of identical restaurants under similar social conditions exist to compare as "controls" to assess the direct effects of inspections. Simply the anticipation of routine inspections probably improves compliance with food safety guidelines and laws (17). The most appropriate mechanism for meas-

uring restaurant sanitation and sharing the results remains a subject of much debate (18–21). Recent regular dissemination of local restaurant scores in print and broadcast media in Tennessee may have increased establishments' attention to addressing deficits. Many businesses may improve compliance with regulations to avoid bad publicity and negative economic repercussions. While no studies have been done to show that these types of negative reproductions have led to decreased foodborne illness in Tennessee or elsewhere, the restaurant inspection system may be an effective mechanism to motivate change within the industry.

Public perception about the relative cleanliness or safety of particular types of restaurants may not reflect reality. Many voluntary interventions, such as strict corporate policies on establishment design, equipment, and hygiene within a particular company can affect a large number of restaurants over a wide geographic area. Such policies and procedures within large multistate corporations are unlikely to be substantially affected by local inspection policies. In contrast, restaurants serving specific ethnic or otherwise easily categorized cuisines are more likely to be locally owned and operated and may be more influenced by local management policies. More systematic assessment of this issue will help focus preventive intervention efforts.

This study suggests that a variety of factors influence the uniformity and reliability of routine restaurant inspections in preventing foodborne disease. Some of these factors might be modified by policies designed to ensure periodic retraining and systematic standardization among inspection evaluations within a jurisdiction. Further evaluating factors important in food safety and how best to control them will be important in improving the system. The Centers for Disease Control and Prevention, in collaboration with the Food and Drug Administration and other agencies, has recently launched an Environmental Health Specialist Network project in seven states. This program will systematically address issues of restaurant inspections and their relationship to food safety and might contribute to our understanding of this system and efforts to improve it.

## Appendix

- "Critical" items on the Tennessee Department of Health food service establishment inspection report<sup>a</sup>
- Food is from an approved source in sound condition, with no spoilage.
- Potentially hazardous food meets temperature requirements during storage, preparation, display, service and transportation.
- Facilities are available to maintain product temperature.
- Unwrapped and potentially hazardous food is not reserved.
- Personnel with infections are restricted from potentially hazardous work.

- Hands are washed according to good hygienic practices.
- Food equipment and utensils are sanitized using appropriate methods.
- Water comes from a safe source, with hot and cold water under appropriate pressure.
- Sewage and waste water disposal are sanitary.
- Plumbing prevents backflow, back-siphonage or dangerous cross-connections.
- Toilet and handwashing facilities are convenient, accessible, well-designed, and appropriately installed.
- There is no detectable presence of insects, rodents, birds, turtles, or other animals and outer openings are protected.
- Toxic items are properly stored, labeled, and used.

“Critical items are violations “which are more likely to contribute to food contamination, illness, or environmental degradation and represent substantial public health hazards and [are] most closely associated with potential foodborne disease transmission” (4).

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# Recombination Resulting in Virulence Shift in Avian Influenza Outbreak, Chile

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Influenza A viruses occur worldwide in wild birds and are occasionally associated with outbreaks in commercial chickens and turkeys. However, avian influenza viruses have not been isolated from wild birds or poultry in South America. A recent outbreak in chickens of H7N3 low pathogenic avian influenza (LPAI) occurred in Chile. One month later, after a sudden increase in deaths, H7N3 highly pathogenic avian influenza (HPAI) virus was isolated. Sequence analysis of all eight genes of the LPAI virus and the HPAI viruses showed minor differences between the viruses except at the hemagglutinin (HA) cleavage site. The LPAI virus had a cleavage site similar to other low pathogenic H7 viruses, but the HPAI isolates had a 30-nucleotide insert. The insertion likely occurred by recombination between the HA and nucleoprotein genes of the LPAI virus, resulting in a virulence shift. Sequence comparison of all eight gene segments showed the Chilean viruses were also distinct from all other avian influenza viruses and represent a distinct South American clade.

**I**nfluenza viruses are segmented, negative-sense, single-stranded RNA viruses of the family *Orthomyxoviridae* and are divided into the genera *Influenzavirus A*, *B* and *C*. However, only type A influenza viruses have been known to cause natural infections of birds. Type A influenza viruses are further divided into subtypes based on antigenic relationships of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. To date, 15 unique HA subtypes (H1–H15) and nine unique NA subtypes (N1–N9) have been recognized. Each virus has one HA and one NA protein, potentially in any combination. Viruses of all HA and NA subtypes with most possible

combinations of the HA and NA subtypes have been isolated from avian species.

Influenza A viruses infecting chickens and turkeys are usually at one of two extremes of virulence. Highly pathogenic avian influenza (HPAI) viruses cause a systemic disease with rapid death in chickens and turkeys, which often approaches 100%. Low pathogenic avian influenza (LPAI) viruses cause a localized infection with little or no disease unless exacerbated by other organisms or poor environmental conditions. To date, all HPAI isolates have been of the H5 or H7 subtypes, although not all H5 or H7 subtype viruses cause HPAI.

Although the virulence of AI viruses for birds is a polygenic trait, one virulence factor is correlated with the hemagglutinin cleavage site. For all influenza A viruses, the hemagglutinin glycoprotein is produced as a precursor, HA0, which requires posttranslational cleavage by host proteases before it is functional and virus particles are infectious (1). All HPAI viruses examined to date have had a motif with multiple basic amino acids (arginine and lysine) at the HA0 cleavage site. In contrast, the cleavage motifs of LPAI viruses typically have only two basic amino acids, at positions –1 and –4 from the cleavage site for the H5 and at positions –1 and –3 for the H7 subtype (2). This difference appears to have a direct influence on viral virulence as LPAI viruses are limited to cleavage by host proteases such as trypsin-like enzymes and are thus restricted to replication at sites in the host where such enzymes are found, i.e., the respiratory and intestinal tracts. Whereas the multiple basic amino acids at the HA0 cleavage sites of HPAI viruses, either as a result of insertion or substitution (2–4), allows the HA0 precursor to be cleavable by ubiquitous host proteases (5). As a result the HPAI viruses are able to replicate systemically, damaging vital organs and tissues, which results in severe disease and death (1).

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Viruses of the H5 or H7 subtype isolated from free-living birds are almost invariably of low pathogenicity for poultry. With the exception of a large die-off of terns in South Africa in 1961 (6), from which A/tern/South Africa/61 (H5N3) was isolated, HPAI virus isolations from free-living birds have been associated with contact with infected poultry, usually as a result of surveillance of birds trapped or found dead on infected poultry farms. In addition, results of phylogenetic studies of H7 subtype viruses indicate that HPAI viruses do not constitute a separate phylogenetic lineage or lineages but appear to arise from low pathogenic strains (7–9). This finding is supported by the *in vitro* selection of mutants virulent for chickens from an avirulent H7 virus (10) and the emergence of HPAI virus from an LP AI virus isolated from swans after repeated passage in chickens (11).

These findings conform to the theories of the molecular basis for the mutation of avian influenza subtype H5 and H7 viruses from low to high virulence in poultry put forward by Garcia et al. (12) and Perdue et al. (13). Essentially they propose that spontaneous duplication of purine triplets results in the insertion of basic amino acids at the HA0 cleavage site and that this occurs due to a transcription fault by the polymerase complex. As pointed out by Perdue et al. (13) this mechanism is clearly not the only means by which HPAI viruses arise, as some appear to result from nucleotide substitution rather than insertion, while others have insertions without repeating nucleotides. Attempts to assess the minimum requirements to confer virulence for H7 avian influenza viruses have used site-directed mutagenesis of the cleavage site of cloned HA followed by *in vitro* expression to examine the effect that progressive amino acid changes at each position of the cleavage site would have upon cleavability (3,14,15). For some H7 influenza viruses, the tetrapeptide motif R-X-R/K-R at the cleavage site leading up to the cleavage point is sufficient for cleavage by furin-like proteases, and variants with cleavage sites that do not conform to this motif are not pathogenic for chickens (3). However, motifs that differ from this standard have been observed from natural outbreaks of H7 HPAI (16).

Between 1959 and the end of 2001, a total of 18 primary outbreaks (10 H7 and 8 H5) of HPAI in poultry were reported (17). However, the geographic distribution of these outbreaks was not uniform across the world (five were in the British Isles; five in Australia; three in areas in Europe other than British Isles; and one each in Pakistan, Hong Kong, Canada, United States, and Mexico). Until 2002, no influenza virus had been isolated in poultry or wild birds in the continent of South America. In May 2002, a LP AI virus of H7N3 subtype was isolated from a broiler breeder flock in Chile, and in June, an HPAI virus of the same subtype was obtained from the same flock (18). We

describe the characterization of the LP AI and HPAI isolates obtained in Chile with particular reference to the HPAI virus, which, while having a 10-amino acid (aa) insert at the HA0 cleavage site, does not conform to the dogma that a -R-X-R/K-R\*G-L-F- motif is a prerequisite for HPAI viruses. We also present evidence that the 10-aa insert present in the HPAI viruses is the result of recombination between the HA and the nucleoprotein genes.

## Materials and Methods

### Viruses

As a result of a disease outbreak in chickens in Chile (18), 13 hemagglutinating isolates (number 176822 obtained in May 2002 and numbers 4322, 4325, 4345, 4346, 4347, 4348, 4418, 4458, 4957, 4966, 4968, and 4977 obtained in June 2002) were submitted to Office of International Epizootics (OIE) reference laboratories for characterization. All viruses were propagated in the allantoic and amniotic cavities of 10- to 11-day-old embryonated specific pathogen free (SPF) chicken eggs for 48 to 72 h at 35°C to 37°C. Viruses submitted as isolates from Chile and other sources were passaged once in SPF eggs after receipt.

### Virus Characterization

Subtype identification was done by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests by using polyclonal chicken antisera against a panel of influenza A reference strains, which had been prepared in SPF chickens. Virulence was assessed by the standard intravenous pathogenicity (IVPI) test by using 6-week-old SPF chickens and the standard pathotyping test by using 4- to 6-week-old SPF chickens (19,20). Both tests give a standard volume of virus intravenously to SPF chickens, but the IVPI test characterizes both illness and how many days the chickens remain alive to produce an index of virulence from 0 to 3. An IVPI index of >1.2 or death in chickens of ≥75% in the standard pathotyping test indicates a highly pathogenic avian influenza virus. Representative low and highly pathogenic viruses were also tested for their ability to replicate in chicken embryo fibroblast cells with or without trypsin (0.25 µg/mL) in the cell culture media.

### Molecular Cloning and Sequencing of Influenza Genes

RNA from the isolates examined in this study was extracted with either the Trizol LS reagent (Invitrogen, Carlsbad, CA), the RNeasy mini kit (Qiagen, Valencia, CA), or the QIAmp Viral RNA mini kit (Qiagen) from infectious allantoic fluid from embryonating chicken eggs before reverse transcription-polymerase chain reaction (RT-PCR) amplification. The RT-PCR amplification was performed with either a one-step or a two-step RT-PCR



reaction. The one-step reaction used the Onestep RT-PCR kit (Qiagen) with incubation steps of 45°C for the PA, PB1, and PB2 genes and 50°C for the other genes for 30 min, and 95°C for 15 min and PCR incubation steps of 30 cycles of 53°C annealing for the PA, PB1, and PB2 genes and 56°C annealing for the HA, NA, M, NP genes for 15 s, 72°C extension for 60 s, and 94°C denaturation for 30 s. For the amplification of the nonstructural and matrix gene segments, primers in the 5' and 3' noncoding region of the RNA segment were used to amplify the complete coding sequence to be used for direct sequencing. The N3 gene segment was RT-PCR amplified in two parts and used for direct sequencing. Electrophoreses was performed on HA, NP, PB1, PB2, and PA PCR products, amplified with specific primers from the noncoding sequence, in a 1% agarose gel, and the products were extracted with the Qiaquick gel extraction kit (Qiagen) and cloned by using the pAMP ligation independent cloning system (Invitrogen). Colonies were screened by using PCR with internal primers; positive cultures were grown overnight, and plasmid was extracted using the Qiaprep spin miniprep kit (Qiagen). Alternatively, a two-step RT-PCR amplification with the Vgen primer (5' AGCAAAAGCAGG) with MMLV reverse transcriptase (Promega, Madison, WI) and PCR with gene-specific primers was performed. For both direct PCR sequencing and plasmid sequencing, the ABI PRISM Bigdye terminator sequencing kit (Perkin Elmer, Foster City, CA) was used, and the reactions were run on ABI 3700 or 310 automated sequencers (Perkin Elmer).

### Sequence and Phylogenetic Analysis

The sequencing information was compiled with the Seqman II program (DNASTAR, Madison, WI), and nucleotide sequences were aligned with sequences from the influenza sequence database with the Megalign program (DNASTAR, Madison, WI) by using the Clustal V alignment algorithm. Pairwise sequence alignments were also performed in the Megalign program to determine sequence similarity between A/chicken/Chile/176822/02 and other published sequences for each gene segment. The origin of a 30-nt insertion at the HA cleavage site was determined by using the best-local-homology rapid search procedure (BLAST) against GenBank sequences. Phylogenetic comparisons of the aligned sequence for each gene segment were generated by using either the maximum parsimony method with 100 bootstrap replicates in a heuristic search with the PAUP 4.0b10 software (Sinauer Associates, Inc, Sunderland, MA) or with the maximum likelihood by using the PHYLIP phylogenetic inference package, version 3.57c (21) with transition/transversion ratios calculated by PUZZLE (22). Sequence data was submitted to GenBank with accession no. AY303630-AY303666.

## Results

### Virus Characterization

All 13 hemagglutinating isolates were identified as influenza A viruses of the H7N3 subtype. Six of the 7 isolates, 4322, 4325, 4418, 4957, 4968, and 4977, tested by the IVPI test were shown to be HPAI viruses with indices between 2.43 and 3.00. An IVPI of >1.2 is classified as a highly pathogenic avian influenza virus. Isolate 176822 was characterized as LPAI with an IVPI index of 0.00. Similar results were observed in the standard pathotyping test. The H7N3 virus was reisolated from chickens that died after intravenous injection with isolates 4322 and 4975. The amino acid sequence of the HA cleavage site was determined and shown to be the same as the injected virus. The low pathogenic and two highly pathogenic Chilean viruses, 176822, 4322, and 4957, were grown in chicken embryo fibroblast cell culture with and without the addition of trypsin to the media. The low pathogenic virus, 176822, did not plaque without the addition of trypsin. The HPAI viruses, which included a representative with and without the additional lysine at the cleavage site (see HA0 sequence below), plaqued with and without the addition of trypsin.

### HA0 Sequence

The deduced amino acid sequence at the hemagglutinin cleavage site for the H7N3 virus of low pathogenicity isolated in May 2002, 176822, was PEKPKTR/GLF. All the HPAI viruses had a 10-aa insert (basic amino acids are underlined) at the HA cleavage site, but the insert varied by a single nucleotide between some of the isolates (PEKPKTCSPLSRCRETR\*GLF [isolates 4322, 4418 and 4977] and PEKPKTCSPLSRCRKTR\*GLF [isolates 4325, 4957 and 4968] that resulted in an aa change from glutamic acid to lysine.

The insertion at the HA cleavage site is unlike any previously reported, and a BLAST search of this nucleotide sequence showed the most closely related sequence in the GenBank database was the nucleoprotein (NP) gene of A/gull/Maryland/704/77 at position 1268–1297, with 28 of 30 nucleotide identities. Nucleotide sequencing of the NP gene of the HPAI Chilean viruses 4077, 4346, 4957 and the LPAI virus 176822 indicated a 100% nucleotide sequence identity of the HA cleavage site insertion with the deduced amino acid sequence CSPLSRRET. Three other conservative amino acid changes in the hemagglutinin were different between the LPAI and all the HPAI viral sequences including at position 38 A>T, position 141 D>N, and position 146 A>T. None of these changes involved potential N-linked glycosylation sites, and no additional potential N-linked glycosylation sites were observed in the Chilean viruses.

### Phylogenetic Analyses

The complete coding sequence for all eight gene segments from the low pathogenic isolate (*A/chicken/Chile/176822/02*) and one of the highly pathogenic isolates (*A/chicken/Chile/4957/02*) was determined. Additional genes from other isolates were also sequenced and used for comparison. The low pathogenic isolate, *A/chicken/Chile/176822/02*, was chosen as the reference isolate for comparison with the influenza database, and pairwise sequence analyses were performed to identify the most closely related isolates for each gene segment. The closest nucleotide sequence similarities ranged from 82.1% for the HA gene to 96.3% for the matrix gene. The amino acid sequence similarity was much higher for all gene segments (Table). Phylogenetic trees were constructed from nucleotide sequences for all eight gene segments, and in general the Chilean isolates clustered most closely with North American avian isolates. Although the most closely related gene segments to the PB2 and PB1 gene segments were viruses isolated from swine, in both cases the swine isolates were thought to be the result of a recently introduced or reassorted avian influenza virus in North America (23,24). Exceptions were the nucleoprotein (Figure 1) and polymerase acid protein (PA) genes, which were most closely related to H7N7 equine influenza viruses which had internal genes derived from the equine type 2 H3N8 viral lineage.

For all gene segments, the Chilean viruses formed a distinct subgroup from other influenza viruses. Analyses of the HA gene indicated that all the Chilean H7N3 viruses were closely related and that the HPAI viruses emerged from the LPAI virus. The H7 phylogenetic tree, when using Equine/Prague/1/56 as the outgroup, shows the avian isolates divided into two main branches that are further subdivided into geographically defined groups (Figure 2). One branch includes the North American avian and the Chilean viruses, and the second branch includes the Eurasian avian and Australian avian viruses. Although the Chilean viruses are most like avian viruses of North American origin they are distinctly different from these viruses and form a unique branch on the tree.

For the N3 tree little sequence data were available to make meaningful observations about the phylogeny. In conserved internal proteins like the matrix and nucleoprotein genes, differences in North American avian and Eurasian avian influenza viruses can be observed at the nucleotide level, but almost all tree topology structure is lost when comparing the same isolates in phylogenetic trees based on amino acid sequence. This observation is common for avian influenza viruses which often have high sequence conservation at the amino acid level, but with large differences at the nucleotide level (25). The results of nucleotide analyses are presented for PB2 and NS genes as unrooted phylograms (Figure 3). Of the genes examined for the six different isolates, little sequence difference was observed between the low pathogenic and highly pathogenic viruses except for the H7 gene segment where an insertion of 30 nucleotides was present at the HA cleavage site.

### Discussion

Sequence analysis shows that the Chilean isolates are unique. None of the eight genes are closely related at the nucleotide level to any other genes in the available sequence databases. For example, the nucleotide sequence of the hemagglutinin gene was 17% divergent from the most closely related virus in GenBank. At the nucleotide level they were more closely related to North American avian or avian-like viruses for six of the eight influenza gene segments. Even the NP and PA genes, which clustered most closely with equine viruses, were more closely related to the North American avian lineage of viruses than any other avian lineage. However, all eight genes were also uniquely distinct with relatively long branch lengths from the most closely related virus. For the H7 hemagglutinin gene, one of the most variable influenza genes, at both the nucleotide and amino acid level, five distinct lineages can be identified, including equine type 1, Eurasian, Australian, North American, and Chilean. The Eurasian and Australian avian viruses are more closely related to each other than the other lineages, but they still differ by

Table. Comparison of individual gene segments of *A/chicken/Chile/176822/02* with influenza viral genes from GenBank with the highest sequence similarity

Gene	Nucleotide similarity	%	Amino acid similarity	%
PB2	Swine/NC/98225/01	82.2	Shorebird/DE/9/96	97.9
PB1	Swine/Ontario/01911-1/99	93.6	Gull/MD/704/77	98.7
PA	Equine/London/1416/73	87.4	TK/MN/833/80	98.3
H7	Chicken/NY/13142-5/94	82.1	Seal/MA/1/80	90.7
NP	Equine/London/1416/73	90.0	Swine/Ontario/01911-1/99	98.4
N3	Turkey/Oregon/71	86.5	Turkey/Oregon/71	93.4
Matrix, M1	Turkey/Oregon/71	96.3	Many Isolates	100.0
M2			Many Isolates	99.0
NS, NS1	Turkey/Canada/63	91.2	Turkey/England/50-92/91	98.3
NS2			Chicken/Mexico/31381-1/94	95.8

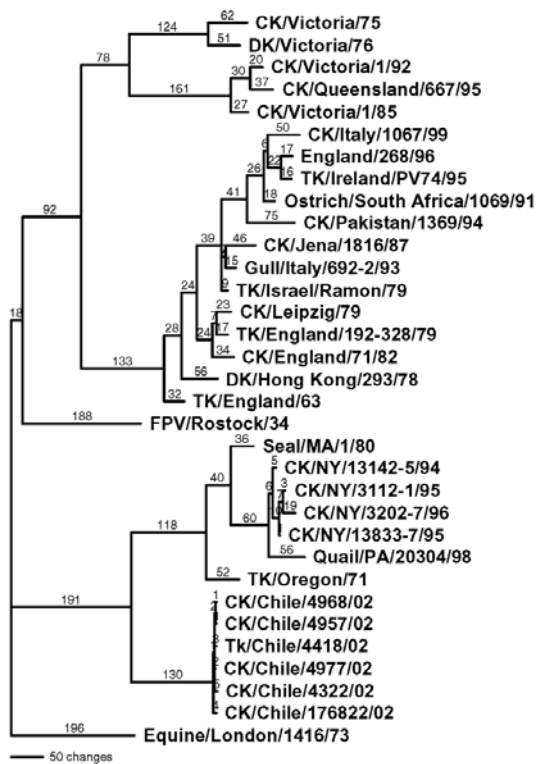


Figure 1. Phylogenetic tree of the hemagglutinin subtype 7 nucleotide sequence, which includes both low pathogenic and highly pathogenic avian influenza viruses from Chile. Representative avian and equine H7 influenza gene sequences are also included. The tree was generated with PAUP 4.0b4 computer program with bootstrap replication (500 bootstraps) and a heuristic search method. The tree is rooted to A/Equine/London/1416/73, and branch lengths are included on the tree. Standard two-letter postal codes are used for states in the United States. TK, turkey; CK, chicken; DK, duck; and FPV, fowl plague virus.

15%–20% at the nucleotide level. A similar relationship exists between the North American and the Chilean lineages, with nucleotide sequence differences of about 20%. For avian influenza viruses, geographic influences rather than the host species infected are usually more important in determining the phylogenetic lineage to which the virus belongs. However, exceptions to this rule of geographic origin have been observed infrequently with avian influenza viruses from wild birds (26).

What is not clear is how prevalent avian influenza viruses are in the wild bird population in South America since avian influenza has not been isolated. This may reflect the low degree of sampling or the low prevalence of infection in wild birds. However antibodies to H1N1 and H3N2 have been reported in wild and domestic birds in Brazil (27).

Phylogenetic analyses of the viruses isolated during the H7N3 outbreak of avian influenza in Chile in 2002 indicate that the HPAI viruses emerged from the LPAI virus or

a close common ancestor. The HA cleavage site motifs of the Chile HPAI viruses do not conform to the recognition motif -R-X-R/K-R\*G-L-F- for the furin-like proteases that are reportedly responsible for allowing HPAI viruses to initiate systemic infections (3). In addition, the insertion of 10 aa could not have occurred by the viral RNA polymerase slippage mechanism proposed by Garcia et al. (12). It seems most likely that this insertion occurred by a RNA recombination event between the HA and NP gene of this virus. The evidence for this is the 100% nucleotide sequence homology between the 30 bases coding for the HA insert and nucleotides 1268–1297 of the NP for the LPAI virus and of the HPAI viruses sequenced. No palindromic sequences were observed on either side of the insert region, so the mechanism by which recombination occurred is not clear. Viruses with the CSPLSRCRKT amino acid insert are most likely to have evolved from those with CSPLSRCRET motif after recombination, since their NP genes only have the latter sequence.

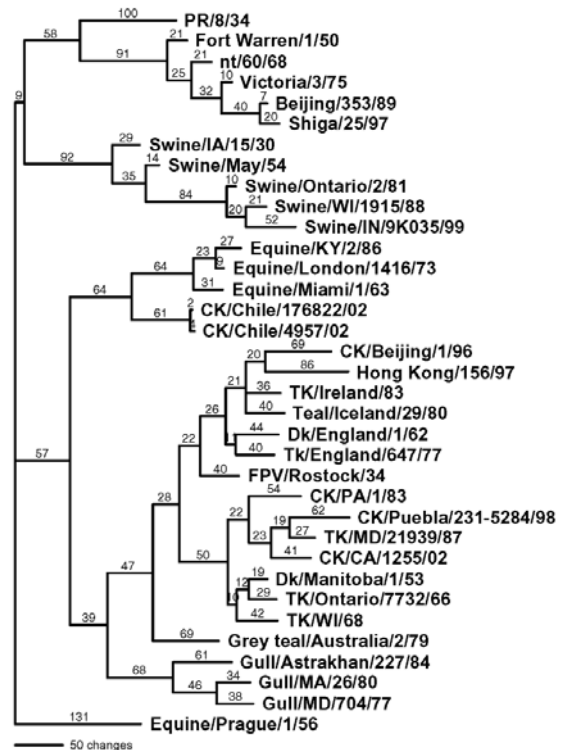


Figure 2. Phylogenetic tree of the nucleoprotein nucleotide sequence, which includes both low pathogenic and highly pathogenic avian influenza viruses from Chile. Representative avian, human, swine, and equine influenza gene sequences are also included. The tree was generated with PAUP 4.0b4 computer program with bootstrap replication (500 bootstraps) and a heuristic search method. The tree is rooted to A/Equine/Prague/1/56, and branch lengths are included on the tree. Standard two-letter postal codes are used for states in the United States. TK, turkey; CK, chicken; DK, duck; and FPV, fowl plague virus. For isolates without a species, it is assumed to be an isolate from a human.

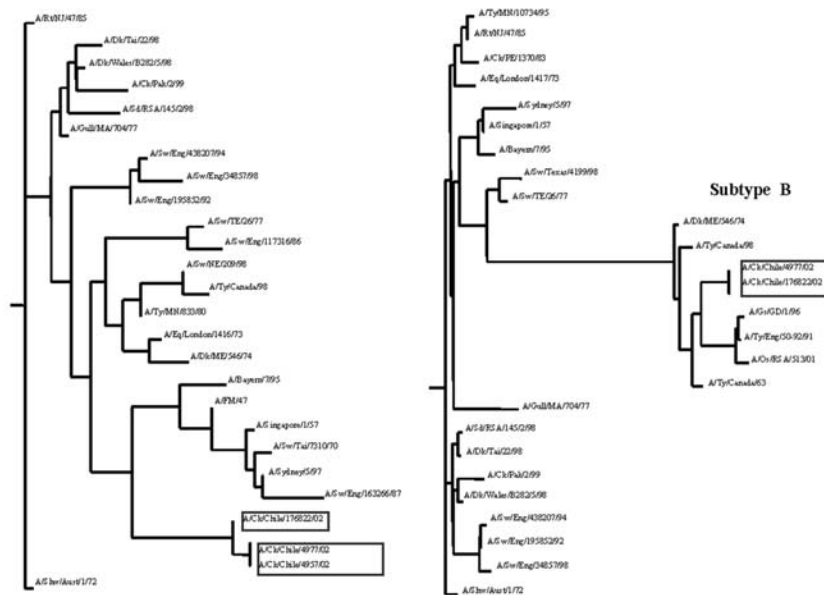


Figure 3. Unrooted phylograms of partial nucleotide sequences of the PB2 and NS genes of selected influenza A viruses including those from poultry in Chile in 2002 (indicated in boxes). Nucleotides 14–188 of PB2 and 50–481 of NS were used for the analyses. The lengths of the horizontal lines are proportional to the number of nucleotide differences.

The increase in virulence between viruses with and without the insert in this outbreak are readily apparent. The virus without the insert caused no illness or death in experimentally infected birds and had an IVPI of 0.0. Viruses with the insert caused severe disease and death in experimentally infected birds and had an IVPI in the range of 2.43–3.0. No correlation with an increased IVPI index was seen with isolates with the additional lysine at the HA cleavage site (range 2.53–3.0). Also, both viruses with the HA cleavage site insert were able to plaque in cell culture without the addition of trypsin to the media, but the virus without the insert could not. All highly pathogenic avian influenza viruses are believed to arise from low pathogenic precursor viruses. The mechanism of this conversion can be extremely variable, but has included both nucleotide substitutions or insertions at the hemagglutinin cleavage site. The Chilean HPAI isolates were unusual not only because of the size of the insert but also because the viruses were highly pathogenic with only three basic amino acids near the cleavage site (–1, –4, and –6 positions) for the first HPAI viruses in the outbreak, although a substitution occurred later in the outbreak, resulting in an additional basic amino acid at the –3 position. Other influenza viruses have been observed with 10 additional amino acids at the HA cleavage site including the equine type 1 (H7N7) viruses, with A/Equine/Prague/56 as the prototype virus. These viruses have four basic amino acids at the cleavage site and can grow in cell culture without trypsin (28), a characteristic for avian viruses of the highly pathogenic phenotype. However, the H7N7 viruses are not considered to cause a systemic disease in horses, but they have been described as causing systemic infection in mice without prior adaptation (29). Also, when the H7

gene from A/Equine/London/1416/73 was reassorted with an avian influenza virus, the reassortant virus had a lethal phenotype in chickens (30).

The two in vitro examples of recombination in avian influenza viruses also involved nucleotide insertions at the hemagglutinin cleavage site. Both cases involved H7 influenza viruses, A/Turkey/Oregon/71 (TK/OR/71) (H7N3) and A/Seal/Massachusetts/1/80 (H7N7). An insert of 54 nucleotides, from 28S host ribosomal RNA, was inserted in A/Turkey/Oregon/71, and 60 nucleotides, from the nucleoprotein gene of the virus, was inserted into A/Seal/Massachusetts/1/80. With in vitro experiments, virus variants of both viruses were selected that could plaque in cell culture without the addition of trypsin, and both showed an increased virulence in chickens. Experimental inoculations resulted in clinical signs suggestive of a systemic disease for A/Seal/Massachusetts/1/80 and a highly pathogenic phenotype for A/Turkey/OR/71 (31,32).

In conclusion, the influenza infections of poultry in Chile in 2002 were both the first reported isolations of influenza viruses in poultry in South America as well as the first HPAI outbreak. The viruses isolated showed several unique properties: 1) They formed a genetic group distinct from other influenza viruses but closest to North American viruses; 2) The HPAI viruses had a unique 10-aa insert at the cleavage site of the HA0 precursor protein; 3) Neither of the two forms of this insert conformed to the assumed minimum motif for high pathogenicity at the cleavage site of –R-X-R/K-R\*G-L-F–; 4) The nucleotide sequence coding for the insert showed 100% homology with a region of the nucleoprotein gene indicating the insertion had occurred as the result of a recombination event.



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# Human Metapneumovirus Infection among Children Hospitalized with Acute Respiratory Illness

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Recent studies have associated human metapneumovirus (HMPV) infection in children with respiratory disease of similar severity as respiratory syncytial virus (RSV) infection. We studied 668 banked swab specimens (one per admission) collected from a population-based, prospective study of acute respiratory illness among inpatient children from two U.S. cities. Specimens were tested for HMPV, RSV, influenza, and parainfluenza viruses by reverse transcription–polymerase chain reaction assays. Twenty-six (3.9%) were positive for HMPV; 125 (18.7%) for RSV; 45 (6.7%) for parainfluenza 1, 2, or 3; and 23 (3.4%) for influenza. HMPV-positive children were significantly older than RSV-positive children. HMPV-positive children required medical intensive care and received supplemental oxygen in similar frequencies to RSV-positive children. Among children hospitalized with respiratory illness, the incidence of HMPV infection was less than RSV, but clinical disease severity mirrored that of RSV infection. Further investigations to better characterize HMPV infection and its clinical effect are needed.

Human metapneumovirus (HMPV) is a recently discovered respiratory pathogen of the family *Paramyxoviridae* belonging to the same subfamily, *Pneumovirinae*, as respiratory syncytial virus (RSV) (1,2). HMPV was first recognized in the Netherlands in 2001 in nasopharyngeal aspirate samples collected from children over a 20-year period (1). It has since been identified in Canada (3,4), Australia (5), the United Kingdom (6), France (7), Hong Kong (8), and the United States (9). Recent reports investigating HMPV have shown that HMPV-infected children who are hospitalized with respiratory illness frequently have clinical diagnoses of bronchiolitis and pneumonia, much like children infected with

RSV (1,4,5,7,10). Clinical symptoms from HMPV-infected children have included nonproductive cough, nasal congestion, and wheezing (5,6,8,11). The most commonly reported abnormality on chest radiography was bilateral infiltrates, indicative of pneumonia (5,11). One child with influenza-like illness seen in an outpatient clinic was reported to have HMPV and negative cultures for influenza virus and RSV (6).

A recent study in Hong Kong looked at HMPV infection by using a prospective, population-based design (8), but no such studies on HMPV infection have been reported that used U.S. data. We present results of such studies and confirm the effect of HMPV infections on acute respiratory illness (ARI) hospitalizations in children.

## Methods

### Study Design

The design of the parent study has been described previously (12). The present study was conducted as part of the New Vaccine Surveillance Network, a population-based active surveillance network for vaccine-preventable diseases related to new vaccines. The study samples were collected at two hospitals in Rochester, New York and three hospitals in Nashville, Tennessee; each cares for >95% of children hospitalized in their respective counties (Monroe County, New York and Davidson County, Tennessee). The children from these two counties provide population-based data. Rochester hospitals also contributed samples from children who resided in six outlying counties. The data from the outlying counties are not regarded as population-based since those counties are also served by their outlying hospitals. Samples were collected from August 2000 through September 2001 from children <5 years of age hospitalized for ARI or ARI-related diagnoses.

Combined nose and throat swab specimens from each child were cultured at the study sites for influenza virus A

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and B; RSV; and parainfluenza viruses 1, 2, and 3. HMPV had not yet been discovered at the beginning of the trial and, therefore, was not investigated. Frozen aliquots of each specimen were analyzed for the above viruses plus HMPV by using reverse transcription–polymerase chain reaction (RT-PCR) assays and GeneScan deoxyribonucleic acid fragment analysis of PCR products. All viruses were confirmed from a second aliquot of sample. A positive virus culture or confirmed RT-PCR was considered a positive test for respiratory viruses other than HMPV.

### RT-PCR Process for HMPV

The RT-PCR assays for HMPV were performed as described previously (13). Briefly, RNA extracts were prepared from 100  $\mu$ L of specimen by using the automated NucliSens extraction system (bioMérieux, Durham, NC). Oligonucleotide primers used for RT-PCR were designed to conserved regions of the HMPV nucleoprotein and fusion protein genes, and one primer for each set was 5'-end-labeled with fluorescein (6-FAM) to facilitate GeneScan analysis. One-step amplification reactions were performed by using the Access RT-PCR System (Promega Corp., Madison, WI). HMPV-positive and -negative controls containing a standardized viral RNA extract and nuclease-free water, respectively, were included in each assay. Amplification products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) with GeneScan software (ver. 3.1.2). Electropherograms were reviewed, and amplification products identified within 2 nt of the expected size (195 nt for nucleoprotein gene; 347 nt for the fusion protein gene) were considered "preliminary" positives. Preliminary positives were then repeat-tested with a new extraction, when an additional specimen aliquot was available. Because viral culture for HMPV was unavailable, specimens were designated "true" positives if the same amplification product for both genes was identified on repeat testing of the sample extract.

### Statistical Analysis

Epidemiologic data were analyzed by using Statistical Analysis Software (SAS) version 8.0 (SAS Institute) and EpiInfo 6.04d (Centers for Disease Control and Prevention, Atlanta, Georgia). Statistical significance was determined by using the chi-square test with  $\alpha = 0.05$ . We include data from the eight counties in all analyses in this paper except for the population-based incidence rates, which include Monroe and Davidson County data only, the principal counties served by the study hospitals. Population-based annual incidence rates per 100,000 children were calculated for a 12-month period, October 2000 through September 2001, by weighting the observed number of enrolled, hospitalized cases to account for sampling

days per week and nonresponse by age, dividing by the 2000 U.S. Census population count, and multiplying by 100,000. Ninety-five percent confidence intervals (CIs) were calculated by bootstrap methods (14).

### Results

Six hundred sixty-eight samples were analyzed from 641 children enrolled in the network from August 2000 through September 2001 (27 children were hospitalized twice and eligible to participate both times). Enrollment by site was evenly distributed (Table 1), and no differences in age or sex distributions of the participants were noted between the sites. However, the Rochester site enrolled significantly more white non-Hispanic children in the study than the Nashville site (risk ratio [RR] = 1.3,  $p = 0.0002$ ), representing differences in overall racial distribution between the sites. More male patients participated in the study than female patients, reflecting the higher proportion of ARI hospitalizations for boys (57%), and the median age of study participants was 6 months (range <1–59 months). Among the 668 samples, 26 (3.9%) tested positive for HMPV; 125 (18.7%) tested positive for RSV; 45 (6.7%) tested positive for parainfluenza viruses 1, 2, or 3; and 23 (3.4%) tested positive for influenza virus.

### HMPV Patient Description

The remaining analyses in this section describe the 26 HMPV-positive patients and compare them with the 125 RSV-positive patients and the rest of the cohort (517 patients who were neither HMPV- nor RSV-positive). The 26 HMPV-positive patients were mostly male (18 [69%]), and 17 (65%) were from the Rochester study site. The median age of HMPV-positive patients was significantly higher than that of the RSV-positive patients (11.5 months and 3 months, respectively;  $p < 0.0001$ ) and approached significance when compared to the median age of the rest of the cohort (11.5 months and 7 months, respectively;  $p = 0.06$ ). Most of the HMPV-positive patients were 6 months to 2 years of age compared with an age of <6 months for most of the RSV-positive patients ( $p = 0.0015$ ) and the rest of the cohort ( $p = 0.0002$ ) (Table 1).

Among the 26 HMPV-positive children, 8 (31%) were reported to have been born >1 month premature, and 16 (62%) were breastfed. Half of the HMPV-positive children were exposed to a household smoker, and eight (31%) attended daycare >4 hours per week. These findings were not significantly different from those for the RSV-positive children or the rest of the cohort (Table 1).

Preexisting medical conditions were assessed both by parent or guardian interviews and medical record reviews. Fourteen (54%) HMPV-positive children had serious underlying medical conditions, compared with 36 (29%) of RSV-positive children (RR = 2.4,  $p = 0.01$ ) and 163 (32%)

## RESEARCH

Table 1. Characteristics of HMPV-positive patients, RSV-positive patients, and the rest of the study cohort, New Vaccine Surveillance Network acute respiratory illness study, August 2000–September 2001

Characteristic	HMPV-positive, n = 26 (%)	RSV-positive, n = 125 (%)	Rest of cohort, n = 517 (%)
Median age	11.5 mo <sup>a,c</sup>	3 mo	7 mo
Range	1–43 mo	<1–56 mo	<1–59 mo
Age distribution			
<6 mo	5 (19) <sup>b,c</sup>	73 (58) <sup>a,c</sup>	244 (47)
6 mo–1 y	8 (31) <sup>b,c</sup>	26 (21) <sup>a,c</sup>	80 (15)
1–2 y	11 (42) <sup>b,c</sup>	19 (15) <sup>a,c</sup>	82 (16)
≥2 y	2 (8) <sup>b,c</sup>	7 (6) <sup>a,c</sup>	111 (22)
Male	18 (69)	72 (58)	295 (57)
Race			
White (non-Hispanic)	17 (65)	75 (60)	256 (50)
Black (non-Hispanic)	8 (31)	32 (26)	172 (33)
Hispanic	1 (4)	13 (10)	55 (11)
Other	0 (0)	5 (4)	26 (5)
Unknown	0 (0)	0 (0)	8 (2)
Underlying medical condition	14 (54) <sup>a,d</sup>	36 (29)	163 (31) <sup>b,d</sup>
Born premature	8 (31)	19 (16)	77 (15)
Breastfed	16 (62)	71 (58)	274 (53)
Exposed to household smoker	13 (50)	50 (41)	236 (46)
Attended daycare	8 (31)	31 (25)	167 (32)
Site			
Nashville	9 (35)	59 (47)	282 (55)
Rochester	17 (65)	66 (53)	235 (45)

<sup>a</sup>Comparison of HMPV-positive to RSV-positive patients. HMPV, human metapneumovirus; RSV, respiratory syncytial virus.

<sup>b</sup>Comparison of HMPV-positive patients to rest of cohort.

<sup>c</sup>p < 0.001.

<sup>d</sup>p < 0.05.

of the rest of the cohort (RR = 2.4, p = 0.02) (Table 1). Among the 14 HMPV-positive children with underlying conditions, the most frequently reported conditions were asthma (8 patients) and heart disease (3 patients). Three children had more than one condition reported.

### HMPV Clinical Description

Admission diagnoses were captured for all patients enrolled in the study. Pneumonia (6 patients), bronchiolitis (6 patients), asthma (3 patients), and fever without localizing signs (3 patients) were the top four admission diagnoses mentioned for HMPV-positive patients. For RSV-positive patients, bronchiolitis (54 patients), RSV (47 patients), pneumonia (33 patients), and asthma (20 patients) were the admission diagnoses most frequently mentioned.

Among the 26 HMPV-positive patients, 24 (92%) had cough as a symptom. This finding was similar to the percentage of RSV-positive patients, but it was a significantly greater percentage than the 64% of the cohort who experienced cough (RR = 1.7, p = 0.0001). Other predominant symptoms of HMPV-positive patients included 19 (73%) with fever, 20 (77%) with nasal congestion, and 21 (81%) with shortness of breath. During the course of their hospital stays, HMPV-positive patients tended to be admitted to intensive care units more than the rest of the cohort (RR =

2.8, p = 0.06), and they were more likely to receive supplemental oxygen while hospitalized (RR = 1.8, p = 0.008) (Table 2).

Twenty-three (88%) HMPV-positive patients received chest radiographs during their hospital stay. This percentage was similar to 84% of RSV-positive patients who received chest radiographs but significantly more than the rest of the cohort, of whom only 59% received chest radiographs (RR = 1.5, p = 0.005). Nine (39%) HMPV-positive patients had radiologic evidence of pneumonia or pulmonary infiltration, while 11 (48%) had evidence of hyperinflation. These percentages were not significantly different from the RSV-positive patients and the rest of the cohort (Table 2).

Up to 10 discharge diagnoses were recorded for each study patient. The most frequently mentioned clinical diagnoses among all discharge codes for HMPV-positive patients were asthma (7 patients), pneumonia (6 patients), bronchiolitis (6 patients), hypovolemia (5 patients), and otitis media (4 patients). These discharges were similar to those coded for RSV-positive patients.

Seasonality for HMPV was estimated by using admission dates for HMPV-positive children. The earliest case appeared in Rochester on January 1, 2001. The first case in Nashville appeared 7 weeks later on February 19, 2001. Though most HMPV cases occurred from January to April



Table 2. Clinical characteristics of HMPV-positive patients compared with RSV-positive patients and the rest of the study cohort, New Vaccine Surveillance Network acute respiratory illness study, August 2000–September 2001<sup>a</sup>

Clinical characteristic	HMPV-positive, n = 26	RSV-positive, n = 125	Rest of cohort, n = 517
	n (%)	n (%)	n (%)
Presenting symptoms			
Cough	24 (92) <sup>b,c</sup>	124 (99)	333 (64)
Fever	19 (73)	85 (68)	383 (74)
Nasal congestion	20 (77)	110 (88)	329 (64)
Shortness of breath	21 (81)	119 (95)	334 (65)
Hospital course			
Admitted to intensive care unit	4 (15)	6 (5)	28 (5)
Received supplemental O <sub>2</sub>	14 (54) <sup>b,d</sup>	76(61)	51 (29)
Intubated	2 (8)	3 (2)	10 (2)
Chest x-ray findings			
	(n = 23)	(n = 105)	(n = 304)
Chest x-ray performed	23 (88) <sup>b,d</sup>	105 (84)	304 (59)
Any pneumonia/infiltration	9 (39)	25 (23)	77 (25)
Hyperinflation	11 (48)	54 (51)	103 (34)

<sup>a</sup>HMPV, metapneumovirus; RSV, respiratory syncytial virus.

<sup>b</sup>Comparison of HMPV-positive patients to rest of cohort.

<sup>c</sup>p < 0.001.

<sup>d</sup>p < 0.01.

2001, Nashville identified one case each in May and in August (Figure).

#### HMPV Population-based Hospitalization Rates

To estimate population-based hospitalization rates, we focused only on cases that resided in Davidson and Monroe Counties. Of the 26 HMPV-positive cases, 19 (73%) resided in these two counties, 9 in Davidson County and 10 in Monroe County. Adjusted for age, the estimated hospitalization rates per 100,000 persons for HMPV infection were 114 cases (95% CI 47 to 198) for children <12 months of age, 131 cases (95% CI 51 to 225) for children 1–2 years of age, and 10 cases (95% CI 0 to 20) for children 2–5 years of age.

#### Discussion

These data suggest that among children <5 years of age hospitalized with respiratory illness, HMPV is associated with a rate of community-acquired ARI similar to that of the combined parainfluenza viruses 1–3 and influenza viruses but substantially less than that associated with RSV. The incidence of 3.9% of positive HMPV specimens among those tested is slightly greater than the 2.3% reported in one Canadian study (10) and slightly less than the 5.5% reported in a Hong Kong study (8) of similar design. Other studies have found that HMPV incidence can vary from year to year, sometimes rivaling or exceeding RSV incidence (13,15). Our single year of data did not allow us to evaluate this.

As reported in other HMPV studies, clinical symptoms of HMPV-positive patients mirrored those of RSV-positive patients. While this comparison could indicate that HMPV may be associated with a similarly severe respiratory ill-

ness such as RSV, the clinical care information is more revealing. The tendency for HMPV-positive patients to be admitted to the intensive care unit and their increased requirement for supplemental oxygen during their hospital stay occurred with similar frequency to RSV-positive patients (Table 2). Therefore, while the incidence of HMPV infection may not compare with that of RSV infection, the disease severity may be very similar.

The observed illness severity could have been influenced by underlying medical conditions reported in half of HMPV-positive patients. Indeed, three of four HMPV-positive children admitted to the ICU and 8 of 14 requiring supplemental oxygen had underlying medical conditions. However, proportions of underlying medical conditions were not significantly different for RSV-positive patients or the rest of the cohort that required these same hospital services.

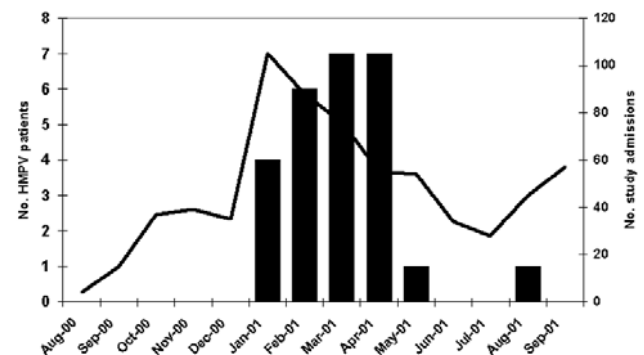


Figure. Distribution of HMPV patients and overall study admissions by month of admission, New Vaccine Surveillance Network acute respiratory illness study, Aug 2000–Sept 2001. Black bars represent HMPV-positive patients, while the line represents study admissions.

Asthma was the most frequently recorded discharge diagnosis among HMPV-positive children. A review of individual cases showed that six of the seven HMPV-positive children with asthma as a discharge diagnosis also had asthma as an underlying medical condition. Two of these children were specifically diagnosed with acute exacerbation of asthma. This finding is similar to findings from a recent HMPV study in Finland that suggested HMPV may stimulate asthmatic episodes in children with asthma (7), but it contrasts findings from an Australian study that showed no such association (16).

Our study found that HMPV-infected children were significantly older (median age 11.5 months) than RSV-positive children (median age 3 months) (Table 1). This age difference was also noted in the Hong Kong study (8), and our median age for RSV-positive patients is consistent with previous reports (17). With other clinical aspects of HMPV infection so similar to RSV, one might have expected the age distribution of HMPV-positive patients to be similar as well. The difference could be due to longer lasting maternal immunity to HMPV compared with RSV, or perhaps the pathogenesis of HMPV disease favors older children. More research is needed to answer these questions.

Our year-round surveillance for respiratory illness showed that HMPV infection tended to occur mainly during the winter months, much like influenza virus and RSV. This finding was also observed in other HMPV studies, though sampling only occurred during the winter months in those studies (1,4,6,9). The identification of HMPV-positive children in May and August, however, suggests that HMPV may continue to circulate in the spring, as was observed in a previous study (13), or possibly year-round.

In the United States, outbreaks of RSV disease tend to occur in the southern United States several weeks before the northeastern United States (18). In this study, HMPV infections were first identified in Rochester 7 weeks before the first infections in Nashville (data not shown). This finding may indicate that HMPV circulation does not exhibit the same pattern of activity as RSV, but our small number of HMPV-positive patients and 1 year of data limit our conclusions. Multiple years of surveillance, encompassing a wide geographic area, are needed to verify this preliminary observation.

Our study is subject to several limitations. The use of combined nose and throat swab specimens, as opposed to nasopharyngeal aspirate specimens, may have resulted in a substantial number of false-negative results. In particular, RSV detection seems to vary substantially by the type of specimen collected (19,20). Whether HMPV detection also varies by specimen type in a similar manner is unknown. Similarly, the use of frozen aliquots of specimens shipped in lysis buffer may have decreased virus yield somewhat

for all the pathogens tested. We may have detected viral RNA from an earlier acute infection but not directly associated with the study illness. The amount and duration of shedding of HMPV has not been well described. Finally, the small number of positive HMPV patients limits our ability to fully characterize features of HMPV infection.

Nevertheless, our data suggest that HMPV infection is associated with severe respiratory illness in children. Further study is now needed to fully explore the link between disease and infection, assess seasonality patterns and incidence of disease, and examine risk factors for severe disease with infection.

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# West Nile Virus, Guadeloupe

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To determine whether West Nile virus (WNV) had reached the archipelago of Guadeloupe, a serologic study in horses and birds was conducted in 2002. Immunoglobulin (Ig) G, IgM, enzyme-linked immunosorbent assay, and seroneutralization tests identified WNV infection in horses and chickens. Six months later, a high rate of seroconversion was observed in horses.

West Nile virus (WNV) was first detected on the American continent during an encephalitis outbreak in birds in New York City in September 1999 (1). Since then, analysis of surveillance data from 2000 to 2002 chronicles the spread of the infection to the South and the West. Infection spread to Florida, Louisiana, and the Cayman Islands in 2001 (2,3) and to northern Mexico in 2002 (4). Resident birds tested positive for WNV in Jamaica in 2002 (5), but the infection has not yet been observed in Lesser Antilles. This division of the French Indies is on the migratory route of wild birds (6), which are the most common vehicles for transmitting the virus over long distances (7). Therefore, a study with the objective of detecting the early appearance of the infection was planned in Guadeloupe on susceptible species (birds and horses) during the summer of 2002.

## The Study

A passive surveillance system of encephalitis in equine and avian species was set up to detect any occurrence of clinical signs of WNV infection. At the same time, a serologic investigation for WNV was conducted in Guadeloupe archipelago. A cross-sectional study was performed on the most susceptible animal species (birds and horses).

The survey on birds was performed in July 2002 on St. Martin/St. Maarten Island (63°5′-18°5′) on domestic ducks (Family: *Anatidae*, *Anas* species), domestic geese (Family: *Anatidae*, *Anser* sp.), and on laughing gulls (*Larus atricilla*), a resident wild species. The French part

of the island belongs to Guadeloupe's archipelago and is located 270 km north of the main island. It is a major resting place for migratory birds, which spend some days or weeks on the ponds before migrating south in the fall (or north in the spring). Therefore, St. Martin was chosen to increase the probability of detecting the earliest serologic conversions on resident birds and to prove the circulation of WNV among resident birds and domestic avian species. A total of 50 ducks and geese from four backyards as well as ducks from the St. Maarten Zoological and Botanical Park were sampled. On a pond, three gulls were caught and released after blood collection.

On Guadeloupe island (61°30′-16°15′), blood samples were drawn from 20 chickens from two different farms neighboring a horse-riding center in December 2002. The survey on horses was planned to be as exhaustive as possible in Guadeloupe and the nearby island of Marie Galante (61°15′-15°55′). Serum samples from 360 of 400 horses thought to live in Guadeloupe were collected in July 2002 (Figure). In locations where positive horses were detected during the first survey, another sampling was drawn from horses from December 2002 to January 2003 to measure the rate of serologic conversion and the incidence of the infection.

Enzyme-linked immunosorbent assays (ELISA) were performed to detect specific immunoglobulin (Ig) G antibodies to WNV in horses, ducks, geese, and chickens. Additional immunocapture IgM ELISA was performed on horses positive for WNV by IgG ELISA (8). Most positive serum samples were tested by plaque reduction neutralization test (PRNT 80) for both WNV and St. Louis encephalitis virus (SLEV).

All the birds (36 ducks, 14 geese, and 3 gulls) sampled from five farms and one pond in St. Maarten at the end of July 2002 tested negative for WNV by IgG ELISA (the ELISA test for the gulls has not been validated). In July 2002, 10 of 360 horses tested positive for WNV by IgG ELISA, and 2 of them were also positive by IgM ELISA. Seropositive horses were located in four different places, two in Guadeloupe and two in Marie Galante.

The results of the survey undertaken in December 2002 to January 2003 in equine centers where positive animals were detected in July 2002 indicated a high rate of WNV seroconversion in horses in these locations (Table 1). In July 2002, the overall WNV prevalence rate (IgG ELISA) was 2.8%, reaching 10.4% in places where infected horses were found (locations A, B, C, D). In January 2003, in these and related places (where some horses were moved from the former areas in July 2002), the prevalence rate increased to 50%. On paired samples, 54 of 114 horses that tested negative in July 2002 were positive in January 2003. This finding represents a seroconversion of 47.4% within 6 months. The incidence rate calculated for the places

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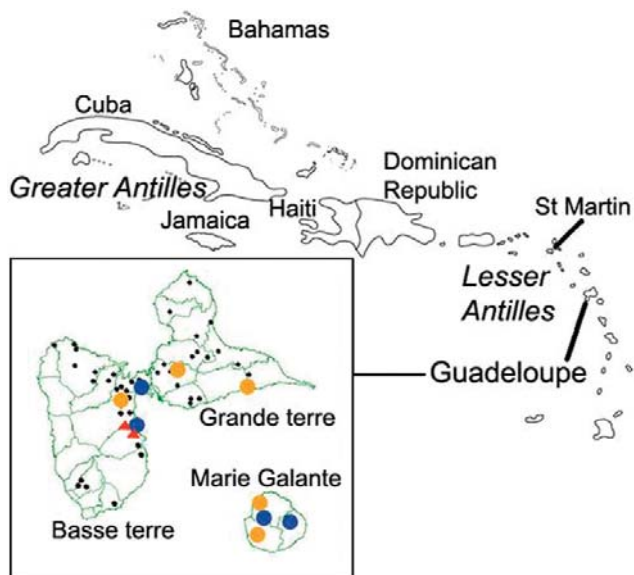


Figure. Location of Guadeloupe archipelago within the Caribbean. Insert: locations of horse and chicken sampling places in Guadeloupe and Marie Galante Islands. Red triangle, farm with seropositive chicken; blue circle, equine center with seropositive horses identified from July 2002; gold circle, equine center with seropositive horses identified from December 2002 to January 2003; black circle, equine center with no seropositive horses.

where outbreaks were noticed (A to I) is 7.9% per month. In December 2002 and January 2003, no IgM antibodies were detected on horses positive by IgG ELISA.

Chickens were collected from two backyards (10 chickens in each place) neighboring one horse-riding club where positive animals were detected. Eleven of these chickens tested positive by IgG ELISA in December 2002.

To confirm the specificity of the results, positive horse samples were tested by PRNT against WNV and SLEV; all the animals showed a higher titer for WNV than for SLEV (Table 2). Specimens from 7 of 10 horses were considered positive for WNV by ELISA. Four chicken serum

samples were tested by seroneutralization and confirmed positive for WNV.

## Conclusions

The serologic survey conducted on horses indicated an active focus of WNV infection in Guadeloupe, probably linked to the first infestation of the archipelago by the virus. The absence of IgM antibodies in horses at the end of 2002 indicates that the seroconversions did not occur during the last weeks of the year but earlier. These results (i.e., the presence of IgM antibodies in 2 of 10 positive animals in July 2002) suggest that the first WNV infections in horses probably occurred during the first 6 months of 2002 and spread in the equine population in the middle of the year.

When birds migrate, they cross the Lesser Antilles (6). A migratory bird, infected before leaving North America or the Caribbean Islands, may develop viremia when reaching St. Martin, Marie Galante, or Guadeloupe islands and transmit the virus to mosquito vectors during the resting period. Migratory birds from the North usually arrive in Guadeloupe later than July; thus, the infection observed in horses in July 2002 was probably not derived from migrating birds that year. WNV was probably introduced into Guadeloupe in the fall of 2001. After one or more introductions, the virus may have gradually spread in the local vector populations and amplified in resident birds even over the winter, when vectors are still active in the Caribbean. Then, 6 months later, the virus spread to susceptible species (horses), in which it was first detected.

In Guadeloupe, both animal and human surveillance systems have been set up and are interacting to detect virus circulation. In that respect, the surveillance of susceptible animal species can provide important indicators for the possible appearance of the disease in humans. As shown in the United States, the death of wild birds is a pertinent indicator for human risk (9). In avian species, mortality and sentinel surveillance has thus been set up. Abnormal

Table 1. West Nile virus IgG<sup>a</sup> antibody prevalence in horses in seven equine centers, Guadeloupe and Marie Galante, July 2002–January 2003

Place	No. tested July 2002	Positive	Prevalence %	No. tested Jan 2003	Positive	Prevalence %	Seroconversion rate %
A	25	1	4.0	23	14	60.9	59.1
B	51	2	3.9	44	27	61.4	59.5
C	7	2	28.6	7	5	71.4	60.0
D	13	5	38.5	12	7	58.3	28.5
E				10 <sup>b</sup>	2	20.0	
F				2 <sup>b</sup>	2	100.0	
G	4	0	0.0	5	3	60.0	60.0
H	7	0	0.0	9	3	33.3	33.3
I	6	0	0.0	24	5	20.8	20.8
Total	113	10	8.8	136	68	50.0	

<sup>a</sup>IgG, immunoglobulin G.

<sup>b</sup>Imported after July 2002.

Table 2. Results of neutralization tests for antibody to WNV and SLEV in serum samples from chickens and horses, Guadeloupe, 2002<sup>a</sup>

Species	Titer to WNV	Titer to SLEV	Interpretation
Chicken	>640	<20	WNV
Chicken	320	<60	WNV
Chicken	160	<40	WNV
Chicken	>640	20	WNV
Horse	160	<20	WNV
Horse	>640	<20	WNV
Horse	40	<20	Flavivirus
Horse	320	<20	WNV
Horse	320	<20	WNV
Horse	40	<20	Flavivirus
Horse	160	<20	WNV
Horse	160	<20	WNV
Horse	<20	<20	Negative
Horse	80	20	WNV

<sup>a</sup>WNV, West Nile virus; SLEV, St. Louis encephalitis virus.

death counts have not yet been observed. This could be related to the absence in Guadeloupe of species known to be extremely susceptible to the infection (Corvidae), vector competence, or the virus strain. A random survey is being implemented on domestic birds to assess the geographic distribution of the infection, in June through July 2003 (beginning of the rainy season), when populations of vectors increase markedly in Guadeloupe. A new serologic prevalence survey in horses is also in process, and clinical surveillance is ongoing. Despite a high rate of WNV-seropositive animals, no clinical disease has been observed. This situation could be related to the virus titer, the rate of infected vectors (which could be too low during the first year after WNV is introduced), or the virus strain. In 2003, mosquito surveillance was implemented in places where deaths in birds or encephalitis cases in horses were observed. Virus detection using reverse transcription-polymerase chain reaction will be used in our laboratory to test dead birds and pools of mosquitoes. These surveys are intended to provide the public health services with distribution and prevalence maps.

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# West Nile Virus and High Death Rate in American Crows

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and Robert Novak†

We document effects of West Nile virus (WNV) on American Crows. More than two thirds of our crows died of WNV infection, peaking when the proportion of infected mosquitoes at roosts was greatest. WNV antibody prevalence in crows was low. Local ecologic effects can be dramatic as WNV inhabits new areas.

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The introduction of West Nile virus (WNV) to North America in the summer of 1999 prompted concern about effects of WNV disease in wildlife. Though the disease has spread rapidly since its introduction, little documentation is available describing the effect of WNV on free-ranging wildlife species. We monitored the emergence and prevalence of WNV infection in American Crows (*Corvus brachyrhynchos*) in Illinois during the spring and summer of 2002; relative to other states, Illinois had the largest number of human West Nile meningoencephalitis cases in 2002 (1).

## The Study

Beginning in February 2002, we captured 156 American Crows with Australian crow traps (2) in Champaign/Urbana in east-central Illinois. Each captured crow was banded, aged by palate coloration (3), measured for sex determination by discriminant function analysis (4), and painted across the span of the tail feathers for identification. Blood samples were taken from all crows to detect antibodies to WNV antigen. Radio transmitters were attached to a subsample of crows. Transmitters were custom designed and weighed <2 g for tail-mount and <3 g for collar-mount, with an expected battery life of 6 months. A tail-mount attachment was used most frequently, modifying the method of Dunstan (5), and individually sized collar-mount transmitters (6) were attached during the molting season. Each crow with a transmitter was tracked at least 10 times per week by vehicle equipped with a receiving system, and locations were stratified by time of day (morning, afternoon, or night). Birds that could not be

located due to transmitter failure, loss, or other reason were searched for with fixed-wing aircraft. This intensive telemetry allowed us to locate dead crows soon after death.

During the months of May through October, radio transmitters were attached to 39 crows. This cohort consisted of 5 adults, 6 sub-adults, and 28 hatch-year crows; 9 were male and 30 were female, as determined by discriminant function analysis or by gonadal observation on dead crows. Of these crows, the fates of 11 birds could not be determined because of transmitter loss, failure, or disappearance. The fates of the remaining 28 birds, comprising 22 females and 6 males, were monitored until death or for the duration of the study (through October). Of the remaining 28 crows, 19 were recovered dead and confirmed positive for WNV with immunohistochemistry (7) or TaqMan reverse transcriptase–polymerase chain reaction (8) yielding a 68% (95% confidence interval [CI] 48% to 84%) WNV-attributed population death rate. The 19 dead crows consisted of 15 females and 4 males; rate of death from WNV did not differ between sexes ( $\chi^2 = 0.0015$ ,  $df = 1$ ,  $p = 0.97$ ), or age classes (Mann-Whitney  $U = 263$ ,  $df = 1$ ,  $p = 0.48$ ). Two crows died and tested negative for WNV during the 6-month observation period of May through October, and 7 birds survived the observation period.

Since our 6-month observation period covered the complete WNV transmission season, no additional WNV deaths would be expected when extrapolating these data to represent a full year. If we assume the number of non-WNV deaths associated with our 6-month study would double in a full year, we extrapolate that the annual death rate for the cohort of 28 crows includes 19 WNV deaths and 4 non-WNV deaths. Therefore, we calculate the annual survival rate of American Crows in east-central Illinois to be 17.9%. This estimate is conservative, as more crows would be expected to die of non-WNV causes in the winter months due to harsher living conditions. The average annual survival rate for breeding-age American Crows from six studies across North America has been estimated at 89.6% (9), though comparing survival rates between studies is difficult as our sample was largely biased towards hatch-year crows.

We also monitored the prevalence of WNV infection in mosquitoes at roost sites associated with our radio-tracked crows. Crows were tracked to six nighttime roost sites continually used throughout the summer. Mosquitoes were collected weekly at each of these sites for 15 weeks using both CO<sub>2</sub>-baited light traps and gravid traps, for a total of 90 trap-nights with each trap type. Throughout the season, we collected 595 pools of mosquitoes representing 10 species, including *Culex (culex) spp.*, *Aedes vexans*, *Anopheles punctipennis*, *A. quadrimaculatus*, *Ochlerotatus triseriatus*, *O. trivittatus*, *Uranotaenia sapphirina*, *Coquillettidia perturbans*, *Orthopodomyia signifera*, and *Culiseta inornata*.

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A pool refers to a uniform grouping of 1–50 mosquitoes collected on the same day at the same location, sorted by species and sex for analysis.

Twenty pools were WNV-positive, with the first positive pool collected the week of July 19, and the last positive pool collected the week of September 20. Of the 20 positive pools, 18 were female and 1 was male *Culex (culex)* spp. and 1 was female *Anopheles punctipennis*. We collected 14 of the positive pools in gravid traps and 6 in CO<sub>2</sub>-baited light traps; positive pools were found at all six roost sites. Combined species minimum infection rates (MIRs) per 1,000 mosquitoes were calculated by week and ranged from 0 to 19. The lowest weekly survival rate of crows occurred from August 16 to September 6 and coincided with the highest MIR in the sampled mosquitoes (Figure).

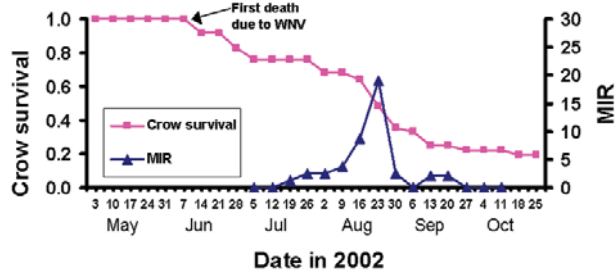


Figure. Survival curve (Kaplan-Meier curve; staggered-entry method) (10,11) for radio-tracked American Crows ( $N = 39$ ) relative to the weekly minimum infection rates (MIR) of mosquitoes collected by week at radio-tracked crow roost sites in east-central Illinois in 2002.

To estimate survival in WNV-exposed crows, we used a blocking enzyme-linked immunosorbent assay (12) to test blood samples ( $N = 156$ ) from all captured crows collected from late February through October in Champaign/Urbana, including the radio-tracked sample. The sample included 13 adults, 13 sub-adults, and 130 hatch-year crows. An inhibition value of  $\geq 30\%$  of monoclonal antibody 3.1112G was required to indicate the presence of WNV-specific antibodies. Inhibition of  $\geq 30\%$  of monoclonal antibody 2B2 served as a confirmatory test, although this antibody is not specific for WNV. Blitvich et al. (12) determined that the most efficient assays for detecting WNV serum antibodies were those that used monoclonal antibodies (MAbs) 3.1112G and 2B2 and that the use of MAb 3.1112G can differentiate between St. Louis encephalitis virus and WNV infections. Serology showed that WNV-specific antibodies were present in 5 of 156 free-ranging crows (Table). One of these seropositive crows was tracked and found dead and positive for WNV 56 days after the blood sample was taken.

Table. Details of the five seropositive crows captured in east-central Illinois, 2002<sup>a</sup>

ID	Bleeding date	Age class	Fate
108	April 26	Adult	Radio-tracked, molted transmitter 19 June
117	June 5	Adult	Radio-tracked, died and negative for WNV 18 June
130	July 9	Hatch-year	Radio-tracked, died and positive for WNV 3 September
180	August 1	Hatch-year	Not radio-tracked
228	August 30	Hatch-year	Not radio-tracked

<sup>a</sup>WNV, West Nile virus.

## Conclusions

This study represents the first peer-reviewed publication describing the death rate from WNV in a tracked wild bird population. While not all infected crows succumbed to WNV, American Crows and other corvids appear to be more differentially susceptible to death due to WNV than noncorvids (13–15). After the arrival of WNV to new areas, crows experience high death rates (16). The relationship between total observed death rate and WNV-attributed death rate in our study indicates a very high level of involvement of WNV in American Crow deaths (90.5%). The proportion of crow deaths attributed to WNV in our study was different from that in other studies in other locations and years. For example, during the 2000 transmission season in the state of New York, 47% of 1,687 dead American Crows tested positive for WNV, with 67% of crows within the epicenter testing positive (15). Surveillance data suggest that the involvement of WNV in the deaths of noncorvid species submitted for WNV testing is much less than involvement of WNV in the deaths of crows ( $\leq 40\%$  in the 2000 New York study [15]).

Our observation of a seropositive crow that died and tested positive for WNV  $< 2$  months after the blood sample was taken merits attention. Similarly, a Red-tailed Hawk that died in the middle of winter raised questions whether the virus could have been acquired earlier, with latent infection later causing death (17). Further investigation of arboviral recrudescence is necessary. Values of MAb inhibition in this crow were just above the threshold for considering a sample positive for WNV-specific antibodies, suggesting a weak response. The antibodies may be due to passive immunity transferred by a parent; however, this crow was around 2.5 months old, and the duration of maternal antibodies may not last this long. The duration of maternal antibodies in crows has yet to be studied.

Experimentally derived death rates of American Crows infected with WNV were 100% in two studies (13,14), in which 8 and 10 infected crows died within 6 and 7 days postinfection, respectively. These studies, combined with our findings, raise concerns about the potential effect of WNV on threatened or endangered corvids, including the



Florida Scrub-Jay (*Aphelocoma coerulescens*), the Hawaiian Crow (*Corvus hawaiiensis*), and the Mariana Crow (*Corvus kubaryi*). As globalization increases and exotic pathogens continue to be introduced, native species will experience new selective pressures with unknown ecologic consequences.

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Ms. Yaremych conducted this research while working towards her master's degree in the Department of Natural Resources and Environmental Sciences at the University of Illinois. She began a Ph.D. program in the Fisheries and Wildlife Department at Michigan State University in fall 2003. Her primary research interests are in wildlife disease ecology and vector-borne pathogen systems.

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# West Nile Virus Encephalitis in a Barbary Macaque (*Macaca sylvanus*)

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An aged Barbary ape (*Macaca sylvanus*) at the Toronto Zoo became infected with naturally acquired West Nile virus encephalitis that caused neurologic signs, which, associated with other medical problems, led to euthanasia. The diagnosis was based on immunohistochemical assay of brain lesions, reverse transcriptase–polymerase chain reaction, and virus isolation.

West Nile virus (WNV) is an arbovirus in the *Flaviviridae* family, which may cause inapparent infection, mild febrile illness, meningitis, encephalitis, and death in birds and mammals, including humans (1–3). Wild birds are the principal reservoirs of WNV, and mosquitoes, especially *Culex* species, are the primary vectors (4). WNV has caused several epidemics in the last 10 years (3). In 1999, WNV was detected in New York City (5). In Canada, WNV was first documented in August 2001 (6). We provide the first report of disease due to naturally acquired infection with WNV in a nonhuman primate.

On August 17, 2002, symptoms of acute neurologic disease were observed in a 25-year-old male, 10.5 kg, Barbary macaque (*Macaca sylvanus*) at the Toronto Zoo. The animal behaved normally when observed by zookeepers the day before. It was housed with 10 other Barbary macaques in an outdoor exhibit with access to indoor housing. The animal exhibited clinical signs of neurologic involvement including ataxia, shaking, a drooping lower lip, excessive salivation, decreased responsiveness to surroundings, and nystagmus. The animal was anesthetized for further evaluation and a limited additional neurologic examination. Minor deviations were noted in hematology and serum biochemistry ranges. Because of severe chronic arthritis and the marked neurologic signs, the animal was euthanized and postmortem examination was performed.

The examination showed moderate gingivitis, generalized severe muscle atrophy, and severe bilateral femorotibial osteoarthritis. Brain and meninges were normal on gross examination. Tissues were collected from all major

organs and frozen at  $-20^{\circ}\text{C}$ . Additional samples from all major organs were fixed in 10% buffered formalin. The fixed brain was cut transversely from the rostral end to the spinal cord, each segment being 0.5 cm to 1.0 cm thick. Representative samples were taken from cortex, thalamus, hippocampus, midbrain, colliculi, pons, obex, medulla oblongata, and cerebellum. The tissue samples were subsequently trimmed and processed for routine histopathology. West Nile Virus immunohistochemistry was prepared by using rabbit polyclonal anti-WNV antiserum (Hana Weingartl, National Center for Foreign Animal Disease, Canadian Food Inspection Agency). Goat anti-rabbit immunoglobulin conjugated to a horseradish peroxidase (HRP)-labeled polymer (EnVision HRP, DAKO Cytomation, Inc., Mississauga, Ontario, Canada) was used as the secondary antibody. Nova Red (Vector Laboratories Canada Inc., Burlington, Ontario) was used as chromogen, and tissues were counterstained with Harris hematoxylin (Fisher Scientific, Toronto, Ontario). For negative controls, nonimmune rabbit serum was substituted for WNV antiserum.

Microscopically, a severe nonsuppurative meningoencephalitis was characterized by generalized gliosis, scattered glial nodules, and perivascular lymphoplasmacytic cuffing. The distribution and severity of lesions were bilaterally symmetrical. The brainstem, pons, colliculi, and cerebellum had the most extensive lesions, the anterior cortex and midbrain were moderately affected, and the posterior cortex mildly affected. Moderate mononuclear infiltrates and edema were present throughout the meninges, most severely over the cerebellum.

Immunohistochemistry for WNV was positive. WNV antigen was present in cerebellar Purkinje cells, neurons, and glial cells within or adjacent to sites of inflammation in the cerebellum, midbrain, and hypothalamus (Figure 1). A few individual glial cells in the cerebral cortex were sparsely stained (Figure 2). Viral antigen was not evident in liver, lymph node, lung, or kidney tissues. Other histopathologic findings included generalized moderate hepatocellular atrophy and focal to diffuse aggregates of lymphocytes, plasma cells, and macrophages, some of which had centers of caseous necrosis, in the renal interstitium.

WNV in brain tissue was also documented by the detection of viral genome by using real-time reverse transcriptase–polymerase chain reaction (RT-PCR) and by virus isolation. Extracted RNA was added to TaqMan PCR reaction (PE Applied Biosystems, Foster City, CA) mixtures containing primers and probes specific for the WNV (North American genotype) envelope gene and the 3' non-translated region (7). Viral RNA was amplified by using an ABI 7700 Sequence detector.

Diluted brain homogenates were used to infect monolayers of Vero cells at 80% confluence. Viral cytopathologic

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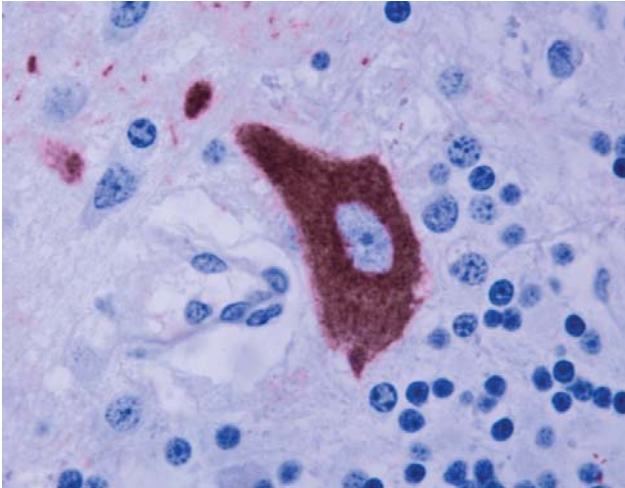


Figure 1. Staining of West Nile virus antigen in the cytoplasm of a Purkinje cell in the cerebellum. Immunohistochemistry. Magnification x40.

changes were observed 3 days post infection. WNV isolation was confirmed by immunofluorescence using chicken anti-WNV polyclonal sera and one-step RT-PCR of infected tissue culture supernatants.

The serum sample was positive for WNV on hemagglutination inhibition (HI) assays (8) with a low titer (1:40). The serum sample was also tested with some of the WNV enzyme linked immunosorbent assay (ELISA) developed for humans. The Centers for Disease Control and Prevention (CDC) immunoglobulin (Ig) M ELISA and the PanBio (Columbia, MD) IgM ELISA produced equivocal results and the PanBio IgG ELISA results were negative.

Thirty-three primates in outdoor exhibits at the zoo were tested for WNV. Serum samples, taken in late December 2002 or spring 2003, were tested for antibodies to WNV by HI assay, and subsequently by plaque reduction virus neutralization (PRVN) if the HI test was positive (8). One of seven olive baboons (*Papio cynocephalus anubis*) had a titer on HI (1:320) and on PRVN (1:80). Two of 16 Japanese macaques (*Macaca fuscata*) had titers on HI (1:160 and 1:20), but only the first of these had a titer on PRVN test (1:40). None of the remaining 10 Barbary apes had serologic reactions.

Clinical signs in our study were similar to those observed in experimentally infected cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) injected intracerebrally with the Egypt-101 strain of WNV, and in rhesus macaques injected intrathalamically by using 10 different strains and mutants of WNV (9,10). Neurologic signs increased for several days and ptosis, paresis of the extremities and sphincters, adynamia, and marked hypothermia were observed. If the animals did not die, their illness went into remission over the following 2 weeks.

The histologic lesions in our studies were similar in morphology findings and distribution to those described in experimentally infected macaques (9,10) and in severe cases in humans (11,12). A number of strains of WNV are capable of long-term persistence in nonhuman primates (10). In these animals the pathogenicity or neuroinvasiveness of the virus decreased with time. Some animals carried the virus for as long as 5 1/2 months, suggesting that primates might be carriers of WNV in foci of infection. These findings should be taken into account when using these animals as sources for cell cultures. A persistently infected nonhuman primate is unlikely to contribute to the WNV-mosquito cycle. Whether a persistently-infected nonhuman primate could be a source of infection for conspecific cage mates or people through bite wounds and scratches is speculative. Persistent infection with WNV has not been reported for other mammals.

The Barbary macaque in this case was probably immunocompromised to some degree, as it was aged and in poor physical condition; however, B-dependent splenic follicles were not atrophic. Hepatocellular and pancreatic atrophy, and poor physical condition, indicated reduced food intake. In humans, increasing age is considered a significant predisposing factor for more severe clinical disease in WNV infection (13).

The prognosis for clinically affected nonhuman primates is difficult to predict based on one affected animal. However, a wide variance occurred in responses in experimentally infected macaques (10). The factors, except increased age, that predispose people to clinical disease are unknown (13). Resolution of the lesions is believed to be complete in human survivors of WNV meningoencephalitis, but for reasons poorly understood, permanent neurologic sequelae occur in some persons (3).

Since most cases in humans are mild or asymptomatic, such is probably the case in nonhuman primates, i.e., the clinically normal seropositives at the zoo. Infection may be

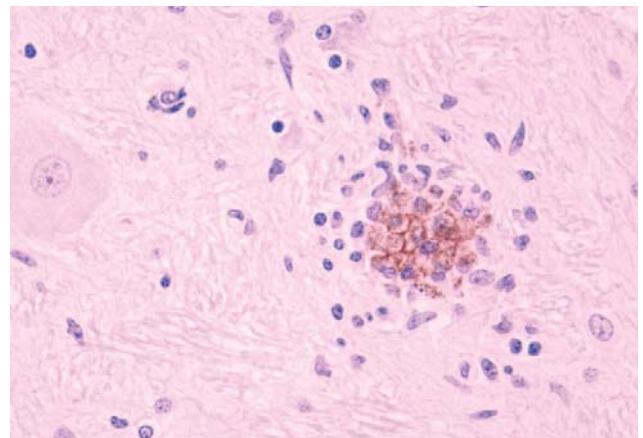


Figure 2. Staining of West Nile virus antigen in a glial nodule in the brainstem. Immunohistochemistry. Magnification x20.



prevalent but subclinical in nonhuman primates housed outdoors, or with access to outdoor holdings, in disease-endemic areas (14). Keeping the animals inside, especially infants and old adults, may be important when the virus is prevalent in surrounding mosquito populations or during periods of peak mosquito activity.

The Barbary ape was the first clinical case of WNV infection in any species recognized at the Toronto Zoo where WNV subsequently caused disease in a variety of avian species. WNV infection should be included as a differential diagnosis in all cases of muscle weakness and neurologic signs in nonhuman primates housed with outdoor access in WNV-endemic areas during the mosquito season. Virologic or serologic confirmation should be obtained in all suspected cases of WNV in nonhuman primates. Since the viremic stage is short, exposure is more reliably confirmed with paired serum samples demonstrating an increase in antibody to WNV (14), usually by HI, confirmed by plaque reduction virus neutralization. Neither of these tests are "species limited" in that they can be used in animals for which specific test reagents are not available (3,15). However, plaque reduction virus neutralization requires biosafety-level 3 facilities. Notably, IgM ELISA tests used in humans gave equivocal or negative results in this case. Laboratory findings and diagnostic techniques used in people are found in several articles (3, 15).

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Dr. Ølberg is a graduate student in zoo and wildlife medicine and pathology at the University of Guelph and Toronto Zoo, Ontario, Canada. His research interests include the use of orally administered anesthetics in non-human primates.

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# Human Case of Lobomycosis

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Stewart Adams,‡ and Richard Kasper\*†

We describe a 42-year-old woman with histologically confirmed lobomycosis, a cutaneous fungal infection rarely reported outside of Latin America. Our case represents the first published report of imported human lobomycosis in Canada and the fifth in an industrialized country.

Cutaneous fungal infections are rarely reported outside of Latin America. We document the first case of imported human lobomycosis in Canada.

## The Study

In February 2001, a healthy, 42-year-old, female geologist from Canada came to her dermatologist with a slowly growing, 1.5-cm diameter, dusky-red, nontender, plaque-like lesion surrounded by keloidal scar tissue on the posterior aspect of her right upper arm (Figure 1). It was located at the site of a scar from a previous excision attempt of a similar lesion 2 years earlier. The original lesion was first noticed while the patient was visiting Southeast Asia in 1996, although she did not seek medical attention until returning to Canada 1 year later. At that time, coccidioidomycosis was diagnosed based on her history of previous travel to a disease-endemic region and on the presence of oval, yeast-like organisms in histologic sections. However, *Coccidioides immitis* was never cultured from the lesion, and serologic studies for this fungus were negative. After the excision in 1997, nothing was noted further until October 1999, when a small lesion, similar in color to the original one, reappeared under the scar and gradually increased in size. The patient had no other skin lesions and was otherwise asymptomatic.

The patient had spent time doing geologic work in various tropical regions over a 7-year period. In 1992–1993, she traveled to the Four Corners region of the United States as well as to California, northern Mexico, and Costa Rica. She lived and worked in the jungles of Guyana and Venezuela for 2 years (1993 and 1994), spending most of her time around the Cuyuni, Essequibo, and Rupununi River areas, although she also spent some time in the Bolivar state of Venezuela. Thereafter, she traveled to Kazakhstan, Indonesia (Irian Jaya), and the Philippines



Figure 1. Photomicrograph of keloidal skin lesion on patient's right upper arm.

(1995 to 1996). During her time in South America, she lived mainly in rural camps and had extensive exposure to freshwater, soil, and underground caves. Health problems encountered during her travels included dengue fever, amebic dysentery, and intestinal helminthiasis. Of note, she had never traveled to the African continent.

Her medical history was otherwise unremarkable. Family history was positive for hypothyroidism. She was a nonsmoker and a social alcohol drinker. She was on hormone replacement therapy but no other medications and had no known allergies. Review of systems was unremarkable. Other than the lesion on her right arm, results of physical examination were normal.

Biopsied tissue specimens of the lesion were obtained and submitted for pathologic and microbiologic examination. The hematoxylin- and eosin-stained tissue sections showed a diffuse, superficial, and deep granulomatous dermatitis with multinucleated giant cells. Intracellular and extracellular unstained fungal cells with thick refractile walls were seen, giving a “sieve-like” pattern to the granulomatous inflammation. The fungal cells stained strongly with periodic acid-Schiff and Grocott methenamine silver stains (Figure 2); cells were spherical or lemon-shaped, approximately 10  $\mu\text{m}$  in diameter, and uniform in size. They were arranged as single cells or in short budding chains joined by narrow, tubelike bridges. Calcofluor white stain (Figure 3) of fresh tissue indicated fluorescent, spherical fungal organisms similarly arranged in chains. The organisms were not cultivatable. Fungal morphology was consistent with *Loboa lobo*. The lesion was completely excised with no subsequent recurrence.

## Conclusions

Lobomycosis is a chronic granulomatous infection of the skin and subcutaneous tissues caused by the fungus *L. lobo* (1,2). It is characterized by the appearance of slowly developing (months, years, or decades), keloid-like, ulcer-

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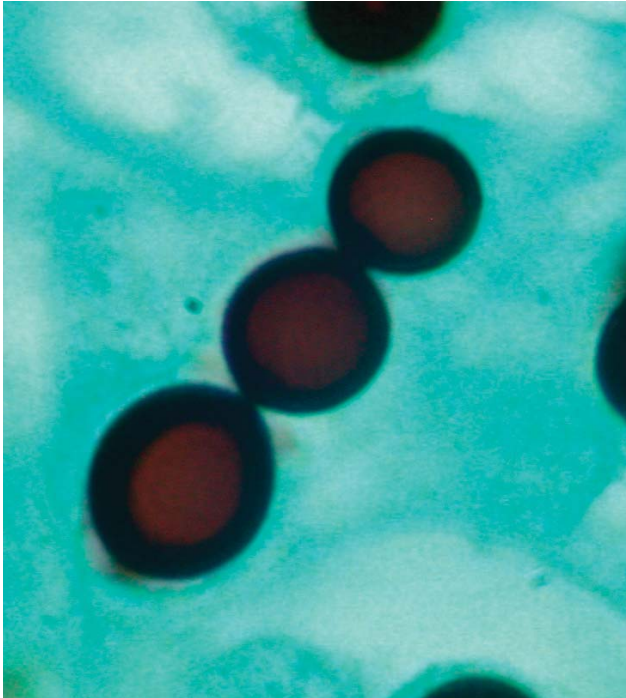


Figure 2. Grocott methenamine silver-stained section of skin biopsy specimen revealing a short chain of lemon-shaped fungal cells connected by thin, tubelike bridges. Morphology is consistent with the appearance of *Loboa lobo*. Magnification x1,000.

ated, or verrucous nodular or plaque-like cutaneous lesions (1,2), usually at a site of local trauma such as from a cut, insect bite, animal bite, or ray sting (1–3). Lesions may be single or multiple and tend to occur on exposed, cooler areas of the body, particularly the extremities and ears (1,2,4). Other sites such as the forehead, face, chest, scapula, lumbosacral spine, buttocks, and scrotum have also been reportedly involved (2–7). Mucous membranes or internal organs are not involved (2,3). Lesions may be non-pigmented, hypopigmented, or hyperpigmented and are usually painless or only slightly pruritic (1,3,4). Little if any local host inflammatory response and no systemic symptoms exist (3,4). In some cases, lesions may spread contiguously or through lymphatic channels, causing cosmetic disfigurement (2,4,5).

On histologic examination, the lesions are composed of dermal granulomas with multinucleated giant cells filled with spherical or lemon-shaped fungal cells 6–12  $\mu\text{m}$  in diameter with doubly refractile walls that are commonly arranged in chains of budding cells connected by thin, tube-like bridges (1,2,6,8). *Loboa lobo* has never been cultivated in vitro (1,2), although it has been successfully transmitted to armadillos, tortoises, and the footpads of hamsters and mice under experimental conditions (2,6).

Natural disease has been described only in humans and in marine and freshwater dolphins (1,2). Lobomycosis was

first described by the dermatologist Jorge Lobo in 1931 (2,3,4,9). His patient was a 52-year-old man who worked as a rubber collector in the Amazonas state of Brazil, who had numerous slowly developing nodular keloidal lesions in the lumbosacral spine area, from which microorganisms resembling *Paracoccidioides brasiliensis* were observed on microscopy (2). Lobo suspected that the patient had a modified form of paracoccidioidomycosis, which he called keloidal blastomycosis (2). A second human case was reported in 1938, after which the disease was termed Lobo's disease (2). More than 500 human cases have been reported to date (3), although the disease appears to be confined to remote tropical areas of South and Central America, especially in communities along rivers (1–3). The natural habitat of the fungus is not known but is believed to be aquatic or associated with soil and vegetation (2,3). Rubber workers, farmers, miners, fishermen, and hunters are particularly at risk due to extensive outdoor exposure (2–4). The condition has been described in Bolivia, Brazil, Colombia, Costa Rica, Ecuador, French Guiana, Guyana, Mexico, Panama, Peru, Suriname, and Venezuela (2–4).

The first report of nonhuman infection occurred in 1971 in an Atlantic bottle-nosed dolphin from Florida (10). In 1973, a dolphin with Lobo's disease was described in Europe along the Atlantic coast of France and Spain (11). The dolphin's caretaker, a resident of Holland, later acquired the disease, the first human case of lobomycosis reported outside of Latin America (11). Other cases of lobomycosis in dolphins have been confirmed, including one in a dolphin off the Texas coast (2,12).

Cases of imported human lobomycosis have been described in the United States and elsewhere (6,7,13,14). A case of lobomycosis involving the chest was recently described in an Atlanta, Georgia, man who had previously

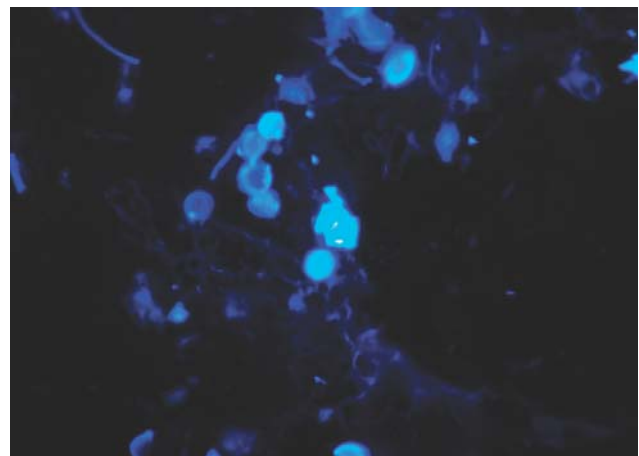


Figure 3. Calcofluor white stain of skin biopsy specimen. A short chain of lemon-shaped fungal cells is seen in the left-center of the field. Cells are connected to each other by thin, tubelike structures. Magnification x400.

traveled to Venezuela and was exposed to extremely high water pressure while walking under Angel Falls (7), although the first published description of a human case in the United States appears to be in an immigrant from Suriname (6). Recently, cases of imported human lobomycosis were reported in France (13) and Germany (14). Other imported cases in industrialized countries are believed to occur but may be misdiagnosed due to physician unfamiliarity with the disease. As far as we are aware, our report is the first of human lobomycosis in Canada. The disease was presumably acquired in Guyana or Venezuela, because her visits to Mexico and Costa Rica were unlikely to put her at risk for infection.

Based on our patient's history, the physical findings, and the histologic appearance of the skin lesion, the diagnosis of lobomycosis was unequivocally made. Although other fungi may resemble *L. loboi* microscopically, including *P. brasiliensis*, *Blastomyces dermatitidis*, and *Histoplasma capsulatum* var *dubiosii* (the cause of African histoplasmosis), none of them form the characteristic chains of fungal cells of uniform size, 6- to 12- $\mu$ m in diameter, connected by thin tubelike isthmuses, the hallmark of lobomycosis (8). Furthermore, unlike *L. loboi*, the other fungi can be grown in vitro on routine mycologic media (2,8). In contrast to *L. loboi*, the mother cell of *P. brasiliensis* forms multiple buds and remains larger than the daughter cells, giving the characteristic "ship's wheel" appearance (1,15,16). In addition, paracoccidioidomycosis is a disease of the oronasal mucous membranes and lungs (1,16). In contrast to *B. dermatitidis*, the fungal cells of *L. loboi* do not form broad-based buds (8). Because the patient had never traveled to Africa, she was not at risk for African histoplasmosis. Other conditions that may clinically resemble lobomycosis include keloids, leprosy, leishmaniasis, chromoblastomycosis, and malignancy (2,4).

Lobomycosis does not usually affect the general health of a person. However, unless lesions are removed at an early stage, the disease persists for life (2). Rarely, squamous cell carcinoma may develop from lobomycotic lesions (2,17).

Successful treatment of lobomycosis usually consists of total surgical excision of the lesion, preferably with wide margins (1,2,9), although adjunctive medical therapy with clofazamine or other agents has sometimes been used in patients with extensive disease, with limited success (2). However, a German patient with lobomycosis had complete clinical and histopathologic resolution of disease after a 1-year treatment course of oral clofazamine and itraconazole (14). One dolphin with Lobo's disease was successfully treated with miconazole (2). Our patient has remained disease-free for more than 2 years after surgical excision of her lobomycotic lesion.

The nomenclature of the fungus has been subject to ongoing debate, although a new genus, *Lacazia*, with *Lacazia loboi* as the type species, was recently proposed by Toborda et al. (16) and appears to be taxonomically valid. The definitive taxonomic status of this fungus awaits in vitro culture and subsequent molecular studies, although phylogenetic analysis of 18S small-subunit rDNA molecules indicates that *L. loboi* is a distinct and novel species phylogenetically close to but fundamentally different from *P. brasiliensis* (15).

In summary, lobomycosis is a slowly progressive, chronic, fungal infection of the dermis that is rarely seen in industrialized countries. This disease should be suspected in patients with single or multiple keloidal skin lesions, particularly if they have traveled to remote areas of Latin America.

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We thank Martin Trotter for taking digital photographs of our pathology slides.

Dr. Elsayed is an associate medical microbiologist for the Medical Staff of Calgary Laboratory Services. He is also an assistant professor of Pathology and Laboratory Medicine and Microbiology and Infectious Diseases at the University of Calgary. His current interests focus on the development of molecular assays to detect and characterize microorganisms and on fungal and mycobacterial infections.

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# Dengue 3 Epidemic, Havana, 2001

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In June 2001, dengue transmission was detected in Havana, Cuba; 12,889 cases were reported. Dengue 3, the etiologic agent of the epidemic, caused the dengue hemorrhagic fever only in adults, with 78 cases and 3 deaths. After intensive vector control efforts, no new cases have been detected.

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In 1994, after 17 years of absence, Nicaragua, Panama, and Costa Rica reported the reintroduction of dengue 3 virus in the region (1,2). The last isolation of this serotype occurred in 1977–1978 in Puerto Rico and Colombia (1,2). Dengue 3 has been related to dengue fever (DF) and dengue hemorrhagic fever (DHF) epidemics. In 7 years the virus has disseminated first to Central-American countries, and later to Mexico, Caribbean countries, and more recently, to South America (3). This virus is genetically different from the dengue 3 strain previously isolated in the Americas and belongs to the same genotype as the virus that caused DHF epidemics in Sri Lanka and India (subtype III) (4). Currently, it is believed that millions of persons in the American region are at risk of dengue 3 infection.

Previously, larger epidemics in Cuba were associated with dengue 1 in 1977 and with dengue 2 in 1981. Both epidemics affected the entire country, producing more than 500,000 and 300,000 dengue cases, respectively. More than 10,000 cases of DHF causing 158 deaths were reported in 1981. From 1982 to 1996, no dengue transmission was reported. In 1997, a dengue 2 epidemic was reported in the municipality of Santiago de Cuba, located in the eastern part of the country (5). In September 2000, a small outbreak of dengue was detected in Havana City; 138

cases of dengue fever (DF) were confirmed at that time, and both dengue 4 and dengue 3 viruses were isolated; the outbreak ended by December (6,7).

## The Study

Havana is the capital city of the Republic of Cuba with 15 municipalities, 2,193,848 inhabitants, and a population density of 3,040/km<sup>2</sup>. Located in the north of the country, it covers an area of 720.84 km<sup>2</sup> and has an annual average temperature of 25°C. La Habana province surrounds Havana City on the east, west, and south. House indexes (percentage of houses with at least one infested container) of 0.05 to 0.91 were reported from 1997 to 2001. In July 2001, house indexes at the municipalities of the city varied from 0.2 to 1.5; however, higher figures were observed at health areas and blocks. These data demonstrate that transmission risk must be assessed in more numerous, smaller geographic areas. The entomologic surveillance and vector control activities involved 4,796 workers; 3,278 family doctors' offices (one family doctor per 120 families and 600 inhabitants) and 81 health areas constitute the primary health care system, and 23 hospitals comprise the second and third levels.

Once the Santiago de Cuba epidemic was detected in January 1997 (5,8,9), an active dengue surveillance system was established throughout the country. Specifically in Havana City, the surveillance was directed at detecting dengue transmission by studying patients with undifferentiated fever and patients with suspected dengue (patients with fever and two or more symptoms of DF such as myalgia, arthralgia, headache, and rash).

A serum sample for dengue immunoglobulin (Ig) M detection was collected 5 days after onset of fever. IgM studies were conducted first at the laboratory of the Centro Provincial de Higiene y Epidemiología de Ciudad Habana (CPHE-CH) by using the ultramicro-enzyme-linked immunosorbent assay ( $\mu$ M ELISA) for dengue IgM detection (10). Positive samples were confirmed at the national reference center, the Tropical Medicine Institute (IPK) by an IgM capture ELISA (11). A comprehensive study from clinical, epidemiologic, and entomologic perspectives was conducted at those health areas where case-patients were found; a second serum sample was collected 2–3 weeks after illness onset to demonstrate the antibody seroconversion or a fourfold increase in antibody titer (12). The Table shows the total number of serum samples studied from 1997 to 2002.

In June 29, 2001, a confirmed dengue case was reported to the national health authorities. The index case had an onset date of June 16. The index case-patient was a 68-year-old white woman who lived in the "26 de Julio" health area of the Playa municipality; she had no history of travel outside the country. The "26 de Julio" health area was a resi-

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Table. Total serum samples from Havana City analyzed at both the provincial (CPHE-CH) and national level (IPK), 1999–2002<sup>a</sup>

Y	CPHE-CH	IPK
1997	807	9,538
1998	1,377	4,794
1999	4,166	10,012
2000	39,335	19,752
2001	65,770	38,513
2002 <sup>b</sup>	11,302	13,304
Total	122,757	95,913

<sup>a</sup>CPHE-CH, Centro Provincial de Higiene y Epidemiología de Ciudad Habana; IPK, Tropical Medicine Institute.

<sup>b</sup>Through March 2002.

dential location with a noncontinuous water supply (it received water every 2 days). The house index was 2.1. Many persons from dengue-endemic countries lived in the area, and many boarding houses also characterized this area.

Within 2 weeks, 20 additional DF cases were serologically confirmed. A retrospective seroepidemiologic study was conducted in a radius of 1 km<sup>2</sup> around the index patient to look for any patients with suspected dengue or undifferentiated fever; 312 febrile patients, and 14 suspected DF patients were found; however, DF was confirmed by serologic studies in 4 of them. All 4 case-patients had dengue IgM and high titers of IgG dengue antibodies. Epidemiologic studies of these patients showed that the first case occurred in late May or early June. The primary case-patient was a 53-year-old white man from the same health area as the index patient.

Once transmission was confirmed, a proactive dengue surveillance program was established, based on information from family doctors. Virologic and molecular surveillance demonstrated that dengue 3 was the etiologic agent of the epidemic. Ninety-one dengue 3 isolates were obtained from samples collected at various times during the epidemic.

Considering the active surveillance and that specimens from all identified clinical case-patients were studied by serologic or virologic methods, the figure of confirmed cases is very close to the total number of dengue clinical cases of the epidemic. All confirmed case-patients were notified. Figure 1 shows the histogram of the epidemic, and Figure 2 shows the municipality distribution and the date of confirmed transmission in the city. By week 30 (July) new cases were detected in the Arroyo Naranjo Municipality, and by the end of October (week 42), almost all municipalities had reported dengue transmission.

The wide clinical spectrum of dengue was established in the Pan American Health Organization (PAHO)/World Health Organization (WHO) guidelines (13). Because of the detection of dengue transmission in the city, the existence of the primary health system, and the strong dengue surveillance system that included laboratories with appropriate technology for serologic diagnosis, we decided to

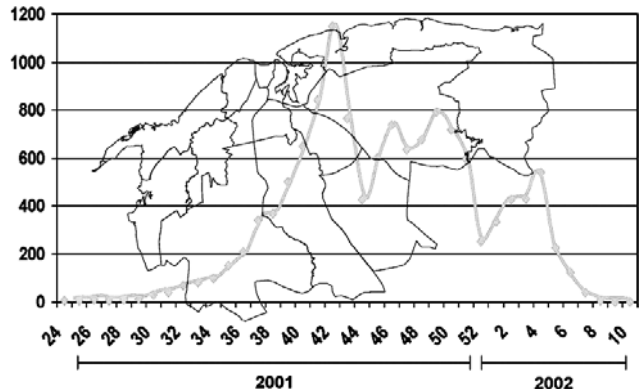


Figure 1. Dengue confirmed case notification according to onset of fever.

extend the clinical, epidemiologic, and laboratory surveillance to the study of almost all undifferentiated fever cases and those patients with a compatible classic dengue picture. A house-by-house survey for febrile cases and dengue suspected cases was performed in Havana City by the family doctors. As a result, 72,162 cases (41,830 undifferentiated fever and 30,332 dengue suspected cases) were epidemiologically, clinically, and serologically studied. Dengue infection was confirmed in 12,889 (17.86%) of the total cases. Of patients with confirmed cases, 1,660 (12.9%) were children and 11,229 were adults (87.1%); 52.4% were female and 47.6% were male. DHF was diagnosed in 78 patients, all adults (16–64 years of age). The main signs and symptoms detected in patients with confirmed dengue cases at the time of hospital admission were fever, 100%; headache, 89%; retroorbital pain, 59.2%; arthralgia, 59.4%; myalgia, 35.2%; and rash, 28.1%. Other symptoms such as cough, diarrhea, nausea, and vomiting were observed in 21.2% of case-patients.

The peak of the epidemic occurred in October and the highest number of cases occurred on October 20 (241

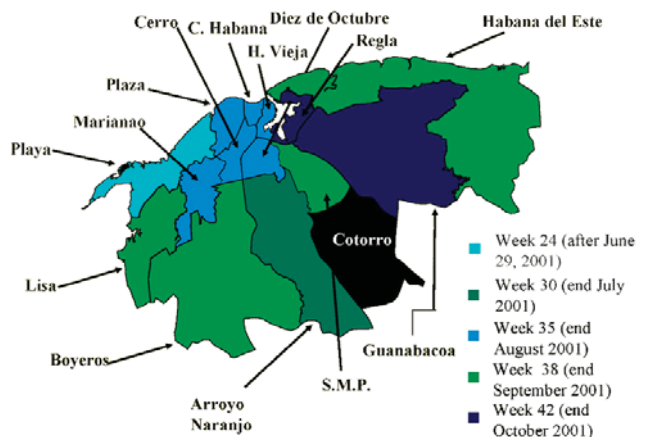


Figure 2. Extension of the epidemic in Havana City, 2001–2002

confirmed cases); 1,150 cases were confirmed by week 42 (October 14–20).

The onset of symptoms of the last two case-patients occurred by February 22, 2002. The epidemic was considered controlled 36 days later with confirmation that no possibility of transmission existed. Case fatality rate was 3.8% among patients with DHF/dengue shock syndrome (DSS). Mortality rate was 0.13/100,000 inhabitants and morbidity rate was 59.2/10,000 inhabitants.

## Conclusions

After the first cases were detected, all patients with suspected dengue and those who were severely ill, or those classified as having DHF/DSS were hospitalized, all adults at the IPK hospital and all children at the Aballi and Cerro Pediatric Hospitals. In total, 4,184 patients were hospitalized, 3,197 adults and 987 children. By the end of the epidemic in January 2002, a broad hospitalization policy was established in areas free of vector, *Aedes aegypti* mosquitoes, (all febrile and dengue suspected case-patients were hospitalized or treated at home with daily visits by the family doctor). (13). Anatomopathologic and histopathologic studies were conducted in all fatal cases.

The vector control strategy had two phases: the first started as soon as the transmission was detected and restricted the number of cases and geographic extension of the epidemic (the risk of expansion of the epidemic was high because of the vector indexes in Havana City and other provinces). The second phase, called the Intensive Campaign, started at the beginning of January 2002 and interrupted transmission and, consequently, lowered the risk of dengue endemicity in approximately 70 days. The Campaign was based on the principles of dengue control established by the PAHO Guidelines (13) with the involvement of the whole community (the head of state, governmental and political bodies at all levels, householders, community organizations, etc.). The objectives of the Intensive Campaign were to control the vector and interrupt dengue transmission. Massive environmental management and sanitation efforts, the elimination of breeding sites, and the elimination of adult mosquitoes were also carried out. These activities were accompanied by extensive efforts to mobilize the community, a strong program of quality control, the active media involvement, and the repositioning of tanks and different water containers. From a house index of 0.49 at the beginning of the Intensive Campaign, this figure diminished to 0.01 by March 4. The intensive active surveillance and the hospitalization of all febrile patients and all patients thought to be infected with dengue were crucial in order to reduce the dengue transmission.

At present, after 17 months since the last dengue case, strong surveillance is maintained by the six regional labo-

ratories and the national reference center, and no additional cases have been reported. These data indicate that endemicity was avoided. Efforts are being made to eliminate *Ae. aegypti* in a regional situation in which the disease has caused unprecedented numbers of cases of DF and DHF (1,015,420 dengue cases and 14374 dengue hemorrhagic fever, with 225 deaths have been reported to PAHO) (data provided by Jorge Arias, WHO American regional office). As has been stated in the PAHO resolution approved by the PanAmerican Health Assembly in September 2002 (14), a concerted action at a regional level is urgently needed.

## Acknowledgments

We thank all doctors, nurses, and personnel of the health system and all persons involved in the epidemic detection and control and elimination of the mosquito vector.

Dr. Peláez is an epidemiologist working at the Centro Provincial de Higiene y Epidemiología de Ciudad Habana, Habana, Cuba. His work focuses on HIV and dengue.

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# Phocine Distemper in German Seals, 2002

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Approximately 21,700 seals died during a morbillivirus epidemic in northwestern Europe in 2002. Phocine distemper virus 1 was isolated from seals in German waters. The sequence of the P gene showed 97% identity with the Dutch virus isolated in 1988. There was 100% identity with the Dutch isolate from 2002 and a single nucleotide mismatch with the Danish isolate.

In the past, fatal morbillivirus infections have been reported in various cetacean and seal species. In pinnipeds, the disease has been described in crabeater seals (*Phoca carcinophagus*) from the Antarctic (1), Baikal seals (*P. siberica* [2]), monk seals (*Monachus monachus*; [3]), Caspian seals (*P. caspica* [4]), and harbor seals (*P. vitulina*) from the North and Baltic Sea (5). Phocine and canine distemper viruses (CDV) were isolated as causative agents in different epidemics of seals. Phocine distemper virus 1 (PDV-1) and CDV represent two distinct but antigenetically and genetically related morbilliviruses (5). An increased number of deaths in the Danish seal population was noticed in May 2002, starting at the Kattegat Isle of Anholt. PDV-1 was isolated as the causative agent (6), and the disease spread to Sweden and Norway in the following month. A second outbreak was observed mid-June in the Netherlands. Subsequently, the disease spread to Germany and Denmark in an eastern direction, and to Belgium, France, Great Britain, and Ireland to the West. In Germany, approximately 7,500 harbor seals died during the epidemic (7). We present morphologic, virologic, and serologic findings in affected seals from German waters.

## The Study

Necropsies of 95 harbor seals (*P. vitulina*) collected from July to December 2002 showed a moderate-to-severe pulmonary alveolar and interstitial emphysema and alveolar edema as the predominant findings. Additional lesions

included mediastinal emphysema, gradually variable suppurative bronchopneumonia, and catarrhalic enteritis. Histologic lesions consisted of interstitial pneumonia with multinucleated syncytial cells and a moderate-to-severe lymphocytic depletion in the lymphoid tissues. Single animals had an acute, focal, nonsuppurative encephalitis (Figure 1A). In addition, neuronal necrosis and mild gliosis were observed. Cytoplasmic and nuclear acidophilic inclusion bodies were detected in respiratory epithelial cells, gastric surface mucous and chief cells, intestinal crypt epithelial cells, and hepatic and pancreatic duct epithelial cells. In the urogenital tract, inclusion bodies were observed in endometrial, vaginal, and epididymal epithelial cells as well as epithelial cells of the renal pelvis and urinary bladder. Occasionally, inclusion bodies were present in neuronal and glial cells of the central nervous system.

Immunohistochemical analyses were performed by using a cross-reacting murine monoclonal antibody specific for the morbillivirus nucleoprotein. Morbillivirus antigen was demonstrated in 39 (45%) of the 86 cases. Morbillivirus antigen was detected in lung, trachea,

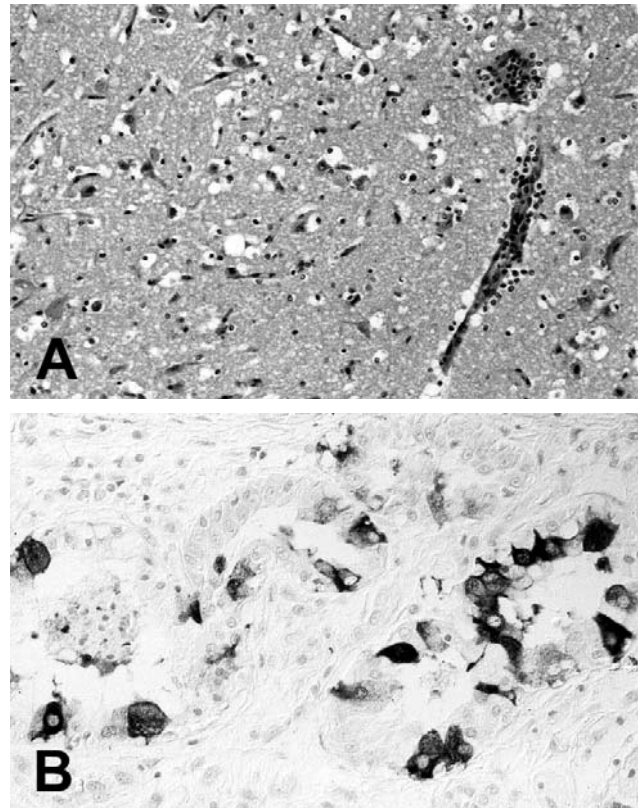


Figure 1. Tissue lesions from a harbor seal (*Phoca vitulina*) with phocine distemper virus infection. (A) Cerebral cortex with nonsuppurative encephalitis. Hematoxylin and eosin staining. (B) Immunohistochemical labeling of morbilliviral antigen in glandular epithelial cells of the lung. Avidin-biotin-peroxidase technique with Papanicolaou's hematoxylin counterstain.

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stomach, intestine, liver, pancreas, kidneys, urinary bladder, female genital mucosa, and epididymal tubules (Figure 1B). In the lymphoid tissues, variable numbers of lymphocytes and macrophages of the follicular and parafollicular areas were positive. In affected areas of the brain, neurons and glial cells contained morbillivirus antigen in the nuclei and cytoplasm.

Screening for morbillivirus-specific nucleic acid in tissue samples from lung, spleen, and lymph nodes as well as in blood samples from 85 seals was performed by reverse transcription–polymerase chain reaction (RT-PCR). For this procedure, universal morbillivirus primers based on the conserved sequence of a 457-bp fragment of the phosphoprotein gene (6,8) were used. PDV-specific RNA was detected in 46 (54%) of the 85 seals from German waters affected from July onward. Both PDV-specific RNA and morbillivirus antigen were detected in 33 (43%) of 77 animals. Seals with no detectable morbillivirus antigen or nucleic acid had pneumonia and endoparasitosis of varying degrees of severity or died of undetermined causes.

Sequence analysis of the RT-PCR product showed an identity of 97% compared to the Dutch isolate of 1988. The German isolate was 100% identical with the PDV isolate from the Netherlands and differed in 1 nt from the Danish isolate (6) (not shown). Phylogenetic analysis showed that the phocine isolates from the two epidemics in European waters formed a discrete cluster, separated from the CDV isolates, including those from lion and Siberian seal (Figure 2).

Neutralization assays using the CDV strain Onderstepoort were performed to determine the titers of serum samples from 187 harbor seals from German waters, collected from 1996 until the outbreak of the epidemic in 2002 (9,10). Because of the cytotoxicity of some serum samples, only titers of  $\geq 10$  were considered positive. No neutralizing antibodies were found in 164 (88%) of 187 serum samples. Titers from 22 (12%) of the 187 animals ranged from 14 to 240 (mean 50.5,  $\pm$  52.6 standard deviation). One animal had a titer of 480.

## Conclusions

The morphologic and immunohistochemical findings in harbor seals from German waters during the recent morbillivirus epidemic in northwestern Europe closely resembled those observed in 1988 (5, 11–13) and confirmed the epithelio-, lympho- and neurotropism of the PDV. The distribution of the viral antigen indicates that the respiratory tract was the primary route of morbillivirus infection. The virus-induced marked lymphoid depletion may have allowed secondary bacterial infections. In contrast to reports about European harbor seals from 1988, no demyelination was detected in seals from German waters in 2002 (5). Whether this finding represents a distinct fea-

ture of the 2002 epidemic or is a result of the small number of investigated animals remains unclear. Seals that died during the morbillivirus epidemic with no detectable viral antigen or nucleic acid may have cleared the virus but still

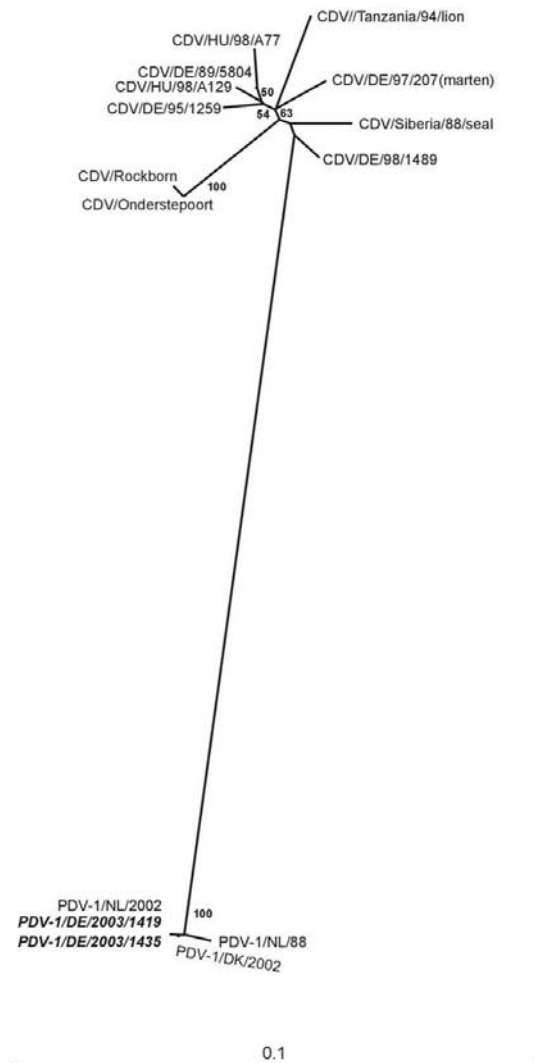


Figure 2. Unrooted neighbor-joining phylogenetic tree constructed by using 369 nt from the gene coding for the morbillivirus P protein. Alignments were calculated with CLUSTAL X (Version 1.8). Bootstrapping (values indicated in %) was performed with 1,000 replicates. TREEVIEW (Version 1.6.5) was used for the graphic display of the tree. The canine distemper virus (CDV) sequences included were from vaccine strains Rockborn (AF181446) and Onderstepoort (AF378705), Siberian seal (AF259551), lion (U76708); CDV isolates originating from dogs 5804/89 (AJ582384), A129/98 (AJ582385), A77/98 (AJ582386), 1489/98 (AJ582387), and 1259/95 (AJ582388); and 207/97 (AJ582389), which was isolated from a marten. Phocine distemper virus (PDV) isolates were PDV-1/NL/88 (AF525289); PDV-1/DK/2002 (AF525287); and PDV-1/NL/2002 (AF525288). The German PDV isolates 1435 and 1419 are in a discrete cluster with a Dutch and with a Danish seal PDV isolate from 1988 and 2002, respectively. Bar, nucleotide substitutions per site.

have virus-induced immunosuppression, which could result in fatal secondary bacterial or parasitic infections. Furthermore, poor preservation of some carcasses may have caused false-negative results. The RNA sequences of the recent virus isolates showed a virus population along the German coast during this epidemic that was almost identical to the isolates from the Netherlands and Denmark in 2002 and that had a high identity to the isolate from 1988 (6). Protective morbillivirus-specific antibody titers were detectable in only a few seals from German waters before the outbreak in 2002, suggesting a high susceptibility for morbillivirus infection in this naive population.

During the morbillivirus epidemic in 1988, approximately 65% of the Dutch, Danish, and German Wadden Sea seal population died (7). The death rate in 2002 is estimated at approximately 51% on the basis of the number of dead seals and the count of the Wadden Sea seal population in 2003 (14). The lower death rate in 2002 may have been influenced by different factors, such as decreased social contacts at the beginning of the epidemic during the late breeding season. In addition, genetic selection of a less susceptible population originating from the survivors of the 1988 outbreak might have resulted in a lower number of deaths during the second epidemic. It remains unclear why both outbreaks started at the Danish Kattgat isle of Anholt. In the past, migrating Arctic seal species, such as harp seals from Greenland, have been suspected as carriers that introduced a morbillivirus into an immunologically naive population (15). This species may have served as a reservoir that maintains the circulation of PDV.

Several epizootics of infectious diseases in marine mammals with increases in air temperature were observed, indicating that environmental influences may have also resulted in the emergence of new epidemics (16). Further studies are needed to determine whether alterations in migration patterns of Arctic seal species caused by changes in climatic conditions are responsible for the two PDV epidemics in northwestern Europe.

Dr. Müller is a research assistant in the Department of Pathology of the School of Veterinary Medicine in Hannover, Germany. Her research activities include a health status monitoring program in seals and cetaceans from the German North and Baltic Sea and cetaceans from the Black Sea with special emphasis on morbillivirus infections.

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# Human Challenge Pilot Study with *Cyclospora cayetanensis*

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We describe a pilot study that attempted to infect human volunteers with *Cyclospora cayetanensis*. Seven healthy volunteers ingested an inoculum of *Cyclospora* oocysts (approximately 200–49,000 oocysts). The volunteers did not experience symptoms of gastroenteritis, and no oocysts were detected in any stool samples during the 16 weeks volunteers were monitored.

*Cyclospora cayetanensis* is a protozoan parasite that may cause gastroenteritis with prolonged, intermittent diarrhea in humans. Characterization and magnitudes of risk factors associated with food and water consumption are unclear because the dose-response relationship and other host-parasite factors for infection with *Cyclospora* are unknown. To characterize infectivity, we performed a study in which inocula of *Cyclospora* oocysts were administered to human volunteers.

## The Study

Stool samples containing high concentrations of *Cyclospora* oocysts and serum specimens were collected from persons with cyclosporiasis in Haiti and the United States. Informed consent was obtained from persons providing the specimens. The Centers for Disease Control and Prevention (CDC) institutional review board (IRB) reviewed specimen collection, informed consent, and patient counseling procedures. Stool specimens were homogenized with water and sieved through cheesecloth. Since *Cyclospora* oocysts must form sporocysts outside the human host before becoming infectious, the filtrate was resuspended in potassium dichromate (2.5%) and shaken for about 3 weeks at room temperature to induce sporulation. After sporulation (67%–94% of oocysts sporulated), samples were stored at room temperature until further processing (2–3 months). Suspensions with the

highest oocyst counts were purified and concentrated by sucrose and cesium chloride gradients (1). See the Table for additional inoculum treatment conditions.

Each candidate inoculum was tested for *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Mycobacterium* spp., *Escherichia coli* O157:H7, enteroviruses, *Hepatitis A virus* (HAV), *Herpesvirus*, *Cytomegalovirus*, *Coronavirus*, *Astrovirus*, *Rotavirus*, *Norovirus*, *Adenovirus*, HIV, *Clostridium difficile* toxin, enterotoxin, and intestinal parasites (data not shown). Serum specimens from the donors of *Cyclospora*-positive stools were tested for serum markers of HIV, HAV, *Hepatitis B virus*, and *Taenia solium*. If serum from a *Cyclospora*-positive stool donor tested positive for any of the above, oocysts from that person were not used. Candidate inocula in which none of these pathogens or toxins were found were used in the human challenge study. This safety-testing protocol was reviewed and approved by both the University of North Carolina School of Medicine IRB and the CDC IRB. Cell culture infectivity assays and animal models were not available to determine the infectivity or viability of the oocysts. Attempts were made to assess the viability of the inoculum by observing sporozoite motility after excystation of the oocysts by different methods. However, these methods did not yield a sufficient number of motile sporozoites to measure viability.

The study was conducted at the General Clinical Research Center at the University of North Carolina Hospital, Chapel Hill, NC. Inclusion and exclusion criteria determined whether a person was eligible to participate in the study. Healthy volunteers were recruited from the University of North Carolina (UNC), Chapel Hill, NC, and the surrounding community. Before enrollment, each candidate received a medical evaluation, and preinoculation serum specimens were collected and archived. The seven study participants comprised four women and three men; three were white, and four were African American. The median age was 26.

After ingesting the inoculum, volunteers were asked to collect all stool specimens for 4 weeks and one specimen a week at weeks 5, 6, 8, and 16. They were also asked to keep a daily record of physical symptoms and the time of each stool passage. In addition, volunteers provided blood and saliva specimens weekly for 6 weeks and at weeks 8 and 16 postdosing. Study outcome measures were a) shedding *Cyclospora* oocysts in stool; b) frequency, weight, color, and consistency of stool; and c) clinical symptoms of gastroenteritis: diarrhea (>3 stools in 24 hours), nausea, vomiting, abdominal pain, myalgia, headache, fever, chills, or fatigue.

Stools were examined to detect oocysts at UNC Chapel Hill. All stool specimens were concentrated by using the formalin-ethyl acetate concentration procedure routinely

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Table. Inoculum treatment and challenge conditions from the *Cyclospora* human challenge study

Volunteer	Oocysts source (no.) <sup>b</sup>	Oocysts ingested, mean ± SD (% sporulated)	Inoculum disinfection <sup>c,d</sup>	Storage medium until dosing	Total storage (mo) <sup>e</sup>	Inoculum ingestion medium and conditions
1	Haiti (3)	647 ± 183 (82)	Bleach and thiosulfate (pre-storage)	Sterile water	6+	Sterile water, then light lunch
2	Haiti (3)	203 ± 22 (69)	Bleach and thiosulfate (pre-storage)	Sterile water	7+	Sterile water, then light lunch
3	Haiti (1)	884 ± 22 (96)	Bleach and thiosulfate (pre-storage)	Sterile water	10	Sterile water, then light lunch
4	MO, USA (1)	4,851 ± 545 (71)	Bleach only; wash on dosing day	Sterile PBS	6+	Sterile water, with a chicken salad meal
5	MO, USA (1)	1,815 ± 249 (67)	Thiosulfate only; wash on dosing day	Sterile PBS	7	Sterile water, with a chicken salad meal
6	MO, USA (1)	4,916 ± 1,153 (67)	Bleach and thiosulfate (pre-storage)	Sterile PBS	7+	Sterile water, with a chicken salad meal
7	GA, USA (1)	48,884 ± 15,345 (94)	Bleach and thiosulfate 3 days predosing	2.5% dichromate	5	Raspberries, then light lunch

<sup>a</sup>MO, Missouri; GA, Georgia; PBS, phosphate-buffered saline.

<sup>b</sup>no., number of stool donors

<sup>c</sup>Household bleach (5.25% sodium hypochlorite) was used for disinfection, and sodium thiosulfate (0.01%–0.1%) was used to inactivate residual bleach in the inocula.

<sup>d</sup>For safety testing laboratory details, contact corresponding author.

<sup>e</sup>Time in storage from stool collection until volunteer dosing date, includes time oocysts were exposed to potassium dichromate in stool until sporulation plus the time the extracted oocysts were in storage media.

used to examine ova and parasites in stool specimens (2). In addition, to increase the sensitivity of detection, all stools from the first 2 weeks postdosing were concentrated by a sucrose floatation procedure (1). Aliquots of the concentrated sample from each procedure were examined microscopically by using a wet mount preparation (2), and 87% of these concentrates were confirmed by a second laboratory at CDC. Criteria to identify *Cyclospora* oocysts were based on size, morphologic characteristics, and ability of the oocysts to autofluoresce under epifluorescence (3).

Inoculum treatment and challenge conditions of this study are described in the Table. Numbers of stools examined per volunteer ranged from 19 to 40; no oocysts were detected in any of the stool samples. Volunteer 1 experienced a brief episode of abdominal cramps on day 7 postdosing. This volunteer attributed the symptom to possible dehydration due to strenuous activity performed in the heat that day. Volunteer 5 produced four loose stools on day 10 postdosing but reported feeling well. This volunteer also had a mildly elevated leukocyte count ( $15.8 \times 10^9/L$ ; normal range  $4.5\text{--}11.0 \times 10^9/L$ ) on day 13 postdosing. However, this volunteer had a mildly elevated baseline leukocyte count ( $13.8 \times 10^9/L$ ) 14 days before dosing. Overall, no conclusive evidence based on clinical or parasitologic diagnostic procedures showed that any volunteers became infected.

## Conclusions

Cyclosporiasis continues to be a difficult emerging infectious disease to understand. Our results are consistent with other researchers' inability to establish *C. cayetanensis* infection in a wide variety of animal models (4). Given these results, questions relating to host susceptibility and

risk factors for infection with *Cyclospora*, the biology of *C. cayetanensis*, survival conditions for *C. cayetanensis* in vitro and in vivo, and factors that allow *Cyclospora* to become infectious in the environment need further study.

Host susceptibility and risk factors for infection are always a consideration when evaluating host response to pathogen exposure. Epidemiologic data suggest that immunity may develop to *C. cayetanensis* in areas where cyclosporiasis is endemic and that the disease is more severe in naïve populations (5). Persons affected in food-borne outbreaks of cyclosporiasis in North America were mostly adults who experienced prolonged symptomatic gastroenteritis, and median food-specific attack rates were high (6,7). In this study, only healthy adult volunteers (22–53 years of age) were recruited from areas in which cyclosporiasis is not known to be endemic. Therefore, although the number of volunteers in this study was small ( $N = 7$ ), epidemiologic data suggest that host susceptibility factors did not substantially contribute to inability of the inocula to cause infection in the volunteers at the doses administered.

Virulence and characteristics of *Cyclospora* necessary to infect human hosts are unknown. Nucleotide sequence variability in the first internal transcribed spacer regions within *C. cayetanensis* from different geographic origins has been observed and suggests the existence of multiple strains (8,9). In addition, data from *Cryptosporidium* human volunteer studies demonstrated that the 50% infectious dose ( $ID_{50}$ ) differed (from 9 to 1,042 oocysts), depending on the isolate used in the study (10). For these reasons, we attempted to vary the inocula by selecting oocysts from persons in different geographic regions (Haiti, Missouri, and Georgia) and increasing the numbers

of oocysts ingested by volunteers (from <1,000 oocysts to approximately 49,000) during the course of this study. Thus, differences in virulence characteristics of *C. cayetanensis* isolates appear not to have been a major factor in failing to establish infection.

All oocysts in stool samples in this study were stored in potassium dichromate (2.5%), and most of the final inoculum preparations were disinfected with bleach (5.25%). *Cryptosporidium parvum* has been stored in 2.5% potassium dichromate (for <6 weeks to >12 weeks) and remained infectious in human volunteers, cell culture, and animals (11,12). Also, baboons inoculated with oocysts never exposed to potassium dichromate were not infected with *Cyclospora* (M. Eberhard, unpub. data). Other parasites of genera related to *Cyclospora* (*Cryptosporidium*, *Eimeria* spp., *Toxoplasma gondii*, sporocysts of *Sarcocystis* spp.) have been shown to resist high levels of bleach (13–15). However, the effects of potassium dichromate and bleach on the *Cyclospora* oocysts used in this study are unknown, since methods to evaluate infectivity and viability were not available.

Naturally occurring *Cyclospora* oocysts may survive for extended periods in the environment, given the marked seasonality of infection in areas where the disease is endemic (6). However, many questions remain about the triggers and conditions necessary for *Cyclospora* oocysts to survive and become infectious in the environment. Given the results of this study, conditions necessary for *Cyclospora* to become infectious were probably not achieved in preparing and storing the oocysts. Future studies are necessary to examine individual and combined effects of temperature, humidity, storage media, and disinfection on the survival, viability, and infectivity of stored *Cyclospora* oocysts. These studies would help determine optimal conditions to stimulate sporulation and maintain infectivity of oocysts in vitro over time. However, such studies will not be possible until suitable cell culture systems or animal host models for cyclosporiasis are developed.

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# Successful Treatment of Human Herpesvirus 6 Encephalomyelitis in Immunocompetent Patient

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and Sylvie Ranger-Rogez\*

We report a case of human herpesvirus 6 (HHV-6) encephalomyelitis in an immunocompetent patient, which was confirmed by viral amplification from cerebrospinal fluid. Cidofovir was used, followed by ganciclovir, because of an adverse effect to probenecid. The patient recovered. HHV-6 should be recognized as one of the causes of encephalomyelitis.

Human herpesvirus 6 (HHV-6) is a member of the *Herpesviridae* family. Like other members of this family, the virus remains in a latent state after primary infection has resolved and can reactivate. HHV-6 encephalomyelitis is an uncommon clinical manifestation in immunocompetent adults. We report the case of a 20-year-old immunocompetent woman who was hospitalized with HHV-6 encephalomyelitis and recovered.

## Case Report

A 20-year-old woman, with no history of medical problems, was admitted to the hospital on February 4, 2002, with a 3-week history of asthenia, myalgia, low-grade fever, urinary retention, and blurred vision. Physical examination showed weakness of all extremities, paresis of her lower limbs, and generalized hyperreflexia. Ocular examination showed a bilateral papillitis and an optic neuritis. The patient was given acyclovir (10 mg/kg, 3x/day) for clinical encephalitis. Despite this treatment, her paresis increased while in the hospital; she was bedridden and unable to sit unsupported. Findings on cranial computed tomographic scan were reported to be normal. Magnetic resonance imaging (MRI) showed a focal lesion in the left thalamus, a medullar cord enlargement, and multiple lesions in the spinal cord white matter. These findings were consistent with inflammatory myelitis but not with multiple sclerosis. The patient did not exhibit any immune abnormalities.

After admission, her first cerebrospinal fluid (CSF) sample was clear with an elevated opening pressure. Its routine analysis indicated 178 leukocytes/mm<sup>3</sup>, with 90% lymphocytes. Total protein and glucose levels were 0.77 g/L and 1.8 mmol/L, respectively. All CSF cultures were negative for bacterial and fungal organisms. The sample was positive for HHV-6 viral DNA by polymerase chain reaction (PCR) (1) by using the primers H6.6 (5'-AAGCTTGCACAATGCCAAAAAACAG-3') and H6.7 (5'-CTCGAGTATGCCGAGACCCCTAATC-3') amplifying a 223-bp target sequence localized on the open reading frame 13 of HHV-6 and followed by hybridization with the 5R probe (5'-CCGTCTTACTGTATCCGAAACAAGTGTCTG-3'), whereas searching for other herpesviruses (i.e., herpes simplex virus type 1 and 2, cytomegalovirus, Epstein-Barr virus, and varicella-zoster virus) and enteroviruses by PCR remained negative. HHV-6 was shown to be A type by a previously described typing method (2).

Because the patient was deteriorating rapidly, she was given a high dose of intravenous methylprednisolone for 5 days. This treatment was not potent. When the diagnosis of HHV-6 encephalomyelitis was established, methylprednisolone was stopped and cidofovir (5 mg/kg for 1 day) therapy was administered. The patient began to recover, and 6 days after this therapy, results of CSF analysis showed 115 leukocytes/mm<sup>3</sup>, with 95% lymphocytes, a protein level of 0.6 g/L, and negative results of HHV-6 amplification. The patient experienced an adverse skin reaction to probenecid given with cidofovir, and the treatment was stopped. On February 27, the patient was still exhibiting neurologic abnormalities, and her CSF was once again positive for HHV-6 by PCR. Intravenous ganciclovir (5 mg/kg twice daily) was then prescribed for 15 days. Within 1 month, the patient had recovered completely, with no sequelae or abnormalities on MRI. One year after the episode of encephalomyelitis, the patient remained free of neurologic defects (Figure).

Several serum samples were taken from the patient on days 2, 12, 22, and 66 after her admission. Serologic tests showed for each serum the same result: anti-HHV-6 immunoglobulin (Ig) G titer of 160 by immunofluorescence assay, accompanied by anti-HHV-6 IgM, except on day 66, showing that the virus had returned to its latent state. The avidity index, measured according to the procedure described by Ward et al. (3), was near 100% in the first three serum samples, suggesting that this episode was a reactivation of an existing viral infection. Serologic tests for HIV were repeatedly negative, as were tests for herpes simplex virus, Epstein-Barr virus (EBV), and cytomegalovirus. HHV-6 PCR performed on peripheral blood mononuclear cells was positive, although it was negative in the serum samples.

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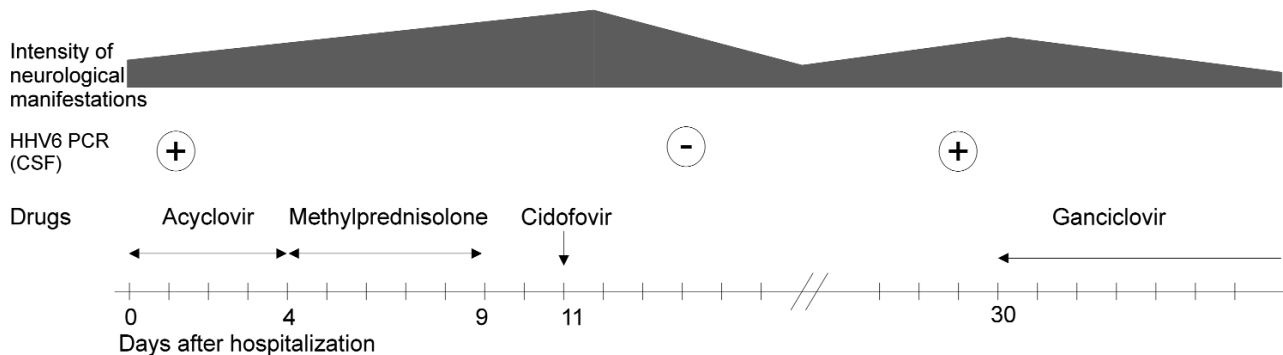


Figure. Clinical and therapeutic course and cerebrospinal fluid analysis. Dosage of acyclovir was 10 mg/kg, 3x/day, methylprednisolone was 1 g/day, cidofovir was 5 mg/kg 1 day, ganciclovir was 5 mg/kg, 2x/day.

The same woman was admitted to the emergency room 1 month after discharge because of dysesthesia of the lower limbs, tonsillitis, asthenia, and low-grade fever. We assumed it could be a novel reactivation of the HHV-6 infection, but this was not confirmed. It was, in fact, an EBV primary infection. One month later, neurologic manifestations had totally disappeared.

### Conclusions

Humans are widely exposed to HHV-6 during childhood, and the seroprevalence is up to 100% in adults. Two types of HHV-6 (A and B) can be identified; no diseases have clearly been linked to HHV-6A infection, whereas HHV-6B is responsible for the childhood disease exanthem subitum. Exanthem subitum complications, including seizures, hemiplegia, meningoencephalitis, or residual encephalopathy, illustrate HHV-6 neurotropism; HHV-6 commonly invades the brain during ES, even in cases of clinically asymptomatic infections. The virus then persists in brain tissues in a latent form (4).

This case is, to our knowledge, the second of encephalomyelitis caused by HHV-6 in an immunocompetent patient. HHV-6 is frequently reported to be implicated in encephalitis or meningoencephalitis in immunocompromised persons, such as HIV-positive patients or transplant recipients, but few reports have implicated HHV-6 in encephalitis in immunocompetent adults (5–7). Our patient was not immunocompromised by either drug therapy or disease. Serologic tests suggested HHV-6 viral reactivation: IgG were present even in the first serum samples, and the avidity index was high. Tests for IgM were positive as well, but anti-HHV-6 IgM can be found during a viral reactivation (8). Symptoms observed were likely to result from a reactivated latent infection of virus in the brain. HHV-6 is known to reactivate frequently during acute infections with other viruses especially with other herpesviruses (9). Although our patient had neither obvious immunosuppression nor any confirmed infection, she may have had a

selective defect in her responses to HHV-6. The virus can invade the central nervous system and, in some cases, cause acute or subacute encephalitis sometimes associated with diffuse or multifocal demyelination (9).

In other cases of neurologic disease induced by HHV-6, such as encephalomyelitis (10), meningoencephalitis (5,6), or encephalitis (7) in immunocompetent adults, patients were treated with acyclovir. Three patients died (6,7,9), and one recovered within 2 days, with small doses of acyclovir (5). Data obtained *in vitro* indicate a greater susceptibility of HHV-6 to cidofovir than ganciclovir or acyclovir (11,12); acyclovir inhibited viral replication only at high concentrations, so our patient was given cidofovir. This regimen had clinical and virologic efficacy, as the patient started to recover and her CSF improved. Viral DNA was not detectable by PCR 6 days after the first injection of cidofovir. Nevertheless, the patient needed other injections of cidofovir to definitively cure the infection, as shown by HHV-6 DNA in her CSF 16 days after the first injection. The second injection was not possible because of a skin reaction to probenecid. The second treatment given to the patient was ganciclovir, which is known to be effective against HHV-6. On this regimen, the patient completely recovered from HHV-6 encephalomyelitis. Because this is a case report and not a controlled clinical trial, we cannot be certain that the antiviral drugs led to her recovery. We note, however, that after cidofovir therapy was stopped, HHV-6 DNA was again detected in the CSF, concurrent with an increase in neurologic symptoms. She began to recover after starting ganciclovir therapy. As a result, we think that the antiherpesvirus drugs led to her recovery.

Of interest is the EBV primary infection that occurred in this patient 1 month after discharge. The immunosuppression induced by HHV-6 probably favored the EBV infection. The paresis observed during this episode was considered a reactivation of the episode during her HHV-6 infection, since paresis is not a classical manifestation accompanying EBV infection,



In conclusion, the case reported here underlines the fact that HHV-6 may cause rapidly multifocal, demyelinating lesions in an immunocompetent adult, even in the case of viral reactivation. Therefore, we think that HHV-6 should be considered in the differential diagnosis of acute demyelinating encephalomyelitis in immunocompetent adults.

Dr. Denes works in the department of infectious diseases in the teaching hospital of Limoges, France, and cares for patients with all types of infections. He is particularly interested in neurologic manifestations of infectious diseases and in bone infections.

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# Ruling Out *Bacillus anthracis*

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Nicholas J. Legakis,\* and Loukia Zerva\*

Optimization of methods for ruling out *Bacillus anthracis* leads to increased yields, faster turnaround times, and a lighter workload. We used 72 environmental non-*B. anthracis* bacilli to validate methods for ruling out *B. anthracis*. Most effective were the use of horse blood agar, motility testing after isolates had a 2-h incubation in trypticase soy broth, and screening isolates with a *B. anthracis*-selective agar.

The potential use of *Bacillus anthracis* as a bioterrorism agent has long been suspected. In 2001, biologic warfare became a reality (1), and microbiology laboratories around the world faced the problem of establishing rapid protocols for ruling out the presence of *B. anthracis* in clinical or environmental samples. To identify *B. anthracis*, both conventional and molecular methods have been applied. Presumptive identification is based on demonstrating a lack of  $\beta$ -hemolysis on sheep (2,3) or horse (3) blood agar plates and the organism's lack of motility (2,3). *B. anthracis*-specific polymerase chain reaction (PCR) assays may also be applied for faster preliminary characterization of isolates. Several protocols targeting chromosomal, pXO1, or pXO2 plasmid sequences have been described (4-9).

Since September 2001, many incidents of alleged bioterrorism have occurred in Greece, as in other countries. Most samples from these incidents are being examined at our Department of Microbiology, National and Kapodistrian University of Athens Medical School. We isolated many bacilli, none of them *B. anthracis*, but encountered difficulties regarding the methods of ruling out *B. anthracis*. We realized that bacilli might produce different hemolysis patterns on blood agar plates made of blood from different species, and that the utility of the *B. anthracis*-selective agar, PLET (polymyxin, lysozyme, EDTA, thallos acetate) (10), was unknown for this type of isolates. We detected occasional false-positive results with PCR protocols previously evaluated by using reference strains rather than field isolates and tried to improve sensitivity of motility detection methods.

Few investigations have dealt with laboratory aspects of the recent bioterrorism attack (9,11,12), and a detailed

evaluation of the methods for ruling out *B. anthracis* has not been reported. We used 72 environmental non-*B. anthracis* bacilli to validate and optimize methods for ruling out *B. anthracis*. We compared blood agar plates made of sheep, horse, and human blood for their ability to demonstrate  $\beta$ -hemolysis and three simple methods for motility detection. We also evaluated the specificity of PLET agar and four previously described PCR assays.

## The Study

During a 12-month period (10/2001-9/2002), 199 consecutive environmental specimens were submitted for possible detection of *B. anthracis*. Seventy-two *Bacillus* spp. strains were isolated; none was *B. anthracis*. Strains were stored at  $-70^{\circ}\text{C}$  and retrospectively analyzed.

Hemolysis types were determined by subculturing them on 5% horse, sheep, and human blood agar plates:  $\alpha$ -,  $\beta$ -, and  $\gamma$ -hemolysis were defined, according to standard criteria (13). Strong  $\beta$ -hemolysis was characterized as hemolysis clearly extending the colony margin, and weak  $\beta$ -hemolysis was characterized by a narrower hemolysis zone or slight hemolysis below colonies. Strains were additionally plated on PLET agar (10). Cultures were incubated at  $35^{\circ}\text{C}$  for 18-24 h in air; blood agar plates were incubated for 48 h. Motility testing was performed by using sterile  $\text{H}_2\text{O}$  at time 0, as well as trypticase soy broth (TSB) at time 0 and after a 2-h incubation at  $35^{\circ}\text{C}$ . Part of a colony was dissolved in  $\text{H}_2\text{O}$  and TSB and examined microscopically (2). Media were supplied from Bioprep (Gerakas, Greece), except for human blood agar plates prepared in house with red blood cell units obtained from blood banks and a blood agar base (Scharlau Chemie, Barcelona, Spain).

Isolates were tested by three *B. anthracis*-specific PCR protocols amplifying a 152-, a 747-, and a 264-bp fragment of the chromosomal Ba813, the *pagA* (pXO1), and the *capC* gene (pXO2) sequences, respectively (5). A PCR recommended by the World Health Organization, which targets a 639-bp sequence of the chromosomal *B. anthracis* S-layer gene, was also performed (8). Crude DNA was extracted by boiling colonies in  $\text{H}_2\text{O}$ . Control strains included the NC08234-03 *B. anthracis* Sterne strain (pXO1+/pXO2-), a *B. anthracis* strain isolated from the malignant pustule of an agricultural anthrax patient, and the *Bacillus subtilis* EO-1 reference strain (kindly provided by the Hellenic Agricultural Ministry). Two microbiologists independently assessed all results. The Fisher exact test was performed for the statistical analysis.

Diverse hemolytic activity was demonstrated by the 72 bacilli on different blood agar plates. At 24 h, strong  $\beta$ -hemolysis was produced by 55 (76%), 41 (57%), and 55 (76%) strains on human, sheep, and horse blood agar plates, respectively, while 6 (8%), 10 (14%) and 7 (10%)

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strains demonstrated weak  $\beta$ -hemolysis (Table). Prolongation of incubation to 48 h resulted in increased detection of strong  $\beta$ -hemolysis on all media.

Both *B. anthracis* strains produced  $\gamma$ -hemolysis on all media at 24 h, except for slight  $\beta$ -hemolysis below areas of confluent growth on human blood agar plates. After 48 h they remained  $\gamma$ -hemolytic on horse and sheep blood agar plates; however, both produced strong  $\beta$ -hemolysis on human blood agar plates.

At time zero of incubation, motility was detected in 33 (46%) and 45 strains (63%) examined in H<sub>2</sub>O and TSB, respectively. All isolates motile in H<sub>2</sub>O were also motile in TSB. The 2-h incubation step in TSB detected another 13 motile strains (total number of motile strains, 58; 81%) and made recognition of motility much easier.

The *capC* and *pagA* gene sequences were not amplified; however, the Ba813 sequence was amplified in seven strains (10%; specificity 90%), and the S-layer sequence was amplified in another two strains (3%; specificity 97%). Twenty-four isolates (33%) grew on PLET; 21 of them were uniformly  $\beta$ -hemolytic. A positive correlation between the ability to grow on PLET and BA813 PCR-positivity was detected. Five out of 24 strains (21%) that grew on PLET were positive by this PCR in comparison with 2 of 48 strains (4%) that did not grow on PLET (Fisher exact test,  $p = 0.037$ ).

## Conclusions

Although other genera are known to produce distinct hemolysis types on different blood agar plates (14), comparative studies for bacilli have not been reported. Most environmental bacilli in our study were  $\beta$ -hemolytic. However, various blood agar plates manifested different abilities to support the expression of  $\beta$ -hemolysis as well as to demonstrate weak and strong  $\beta$ -hemolysis. In that respect, it is important to underline that weak  $\beta$ -hemolysis is interpreted with caution; isolates will be incubated for another 24 h or considered nonhemolytic strains.  $\beta$ -hemolysis was easier to recognize on all media after 48 h, but sheep blood agar plates were the least effective medium in detecting  $\beta$ -hemolysis. Finally,  $\beta$ -hemolysis results

obtained with horse and human blood agar plates, although not identical, were usually in agreement and differed from those obtained with sheep blood agar plates (data not shown).

The production of strong  $\beta$ -hemolysis on human blood agar plates by the *B. anthracis* strains was unexpected, as this organism has been considered traditionally non-hemolytic. Recently, however, the ability of *B. anthracis* to express  $\beta$ -hemolysis was reported (15,16). Broth culture supernatants possessed hemolytic activity against human and sheep erythrocytes, whereas richness of media affected hemolysis expression (15). Another study demonstrated the induction of strong  $\beta$ -hemolysis on human but not sheep blood agar plates under anaerobic conditions (16). In fact, a study conducted in 1957 reported 45  $\beta$ -hemolytic strains among 120 *B. anthracis* isolates that had been cultured on rabbit blood agar plates (17). Therefore, withholding the use of human blood agar plates would be prudent; horse blood agar plates should be used as the most "informative" medium.

According to our findings, the 2-h incubation of bacilli in TSB greatly improves recognition of motile strains. Increased motility detection using TSB rather than H<sub>2</sub>O has been demonstrated with 12 non-*B. anthracis* strains (18), though test performance after incubation was not assessed. Apparently, motile bacilli become immobilized in distilled water, while the practice of incubating them in a rich broth until they reach exponential growth phase was actually recommended in the past (19).

All bacilli tested negative for the presence of *capC* and *pagA* sequences, but seven strains were positive for Ba813. All were strongly  $\beta$ -hemolytic and five were motile. These results prompted us to assess the specificity of the S-layer PCR, which, to our knowledge, has not been evaluated before. Only two strains, different from the above seven, were positive: a motile, strongly  $\beta$ -hemolytic strain and a nonmotile,  $\gamma$ -hemolytic strain.

With the exception of the Laboratory Response Network real-time PCR (9), the specificity of other PCR protocols (4–8,20) has not been evaluated before by testing field isolates from suspected bioterrorism incidences.

Table. Numbers (%) of environmental *Bacillus* spp. isolates exhibiting various types of hemolysis on human, sheep, and horse blood agar plates at 24 and 48 hours of incubation

Type of hemolysis	Incubation time											
	24 h						48 h					
	Human <sup>a</sup>		Sheep <sup>a</sup>		Horse <sup>a</sup>		Human		Sheep		Horse	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
$\alpha$	1	(1.4)	5	(6.9)	0	(0)	0	(0)	0	(0)	1	(1.4)
$\gamma$	10	(13.9)	16	(22.3)	10	(13.9)	4	(5.6)	9	(12.5)	2	(2.8)
Total $\beta$	61	(84.7)	51	(70.8)	62	(86.1)	68	(94.4)	63	(87.5)	69	(95.8)
Strong $\beta$	55	(76.4)	41	(56.9)	55	(76.4)	65	(90.3)	55	(76.4)	66	(91.6)
Weak $\beta$	6	(8.3)	10	(13.9)	7	(9.7)	3	(4.1)	8	(11.1)	3	(4.2)
Total	72		72		72		72		72		72	

<sup>a</sup>Human, sheep, and horse refer to the respective blood agars.

False-positive results have been reported with other *cap* sequence PCR assays (4,6), while efforts to establish a specific chromosomal assay have been frustrating (6,7,20). As *B. anthracis* strains cured from one or both plasmids exist naturally or may be obtained in vitro (3), and false-negative results may be encountered with plasmid-specific PCR assays (4), the availability of a chromosomal PCR is desirable. Our results indicate that to preserve the positive predictive value of the evaluated molecular tests, chromosomal assays should always be performed in conjunction with plasmid PCR.

Because environmental and, to a lesser extent, clinical samples may be heavily contaminated, a selective medium for *B. anthracis* would be useful. PLET is used in environmental investigations of agricultural anthrax outbreaks (3), because it inhibits other bacilli and gram-negative rods (10). In our study, the specificity of PLET was low, but PLET is still valuable, because by inhibiting two thirds of contaminating bacilli as well as other bacteria, background will decrease and isolating colonies will be easier and faster. However, the characteristic colony morphology of the two *B. anthracis* strains on blood agar was not reproduced on PLET. Examination of a large number of *B. anthracis* strains is required to confirm these observations.

A positive correlation was detected between the ability to grow on PLET and Ba813 PCR-positivity. Of note, non-*B. anthracis* strains previously reported to be Ba813 PCR-positive were isolated by using PLET (20). Ba813-positive bacilli may be very closely related to *B. anthracis* and demonstrate, therefore, phenotypic similarities like the ability to grow on PLET.

In conclusion, horse blood agar plates provide better recognition of  $\beta$ -hemolysis, and testing after a 2-h incubation in TSB improves motility detection. The application of these tests along with PLET agar will have a substantial impact on public health laboratories that process large numbers of specimens. Workload will decrease, and the presence of *B. anthracis* will be ruled out faster, leading to earlier termination of chemoprophylaxis and diminished anxiety of exposed persons. Standardization and validation of molecular assays as direct detection tests will further decrease turnaround time; however, these methods only complement conventional testing. Selective or differential media and further refinement of conventional techniques will still be needed.

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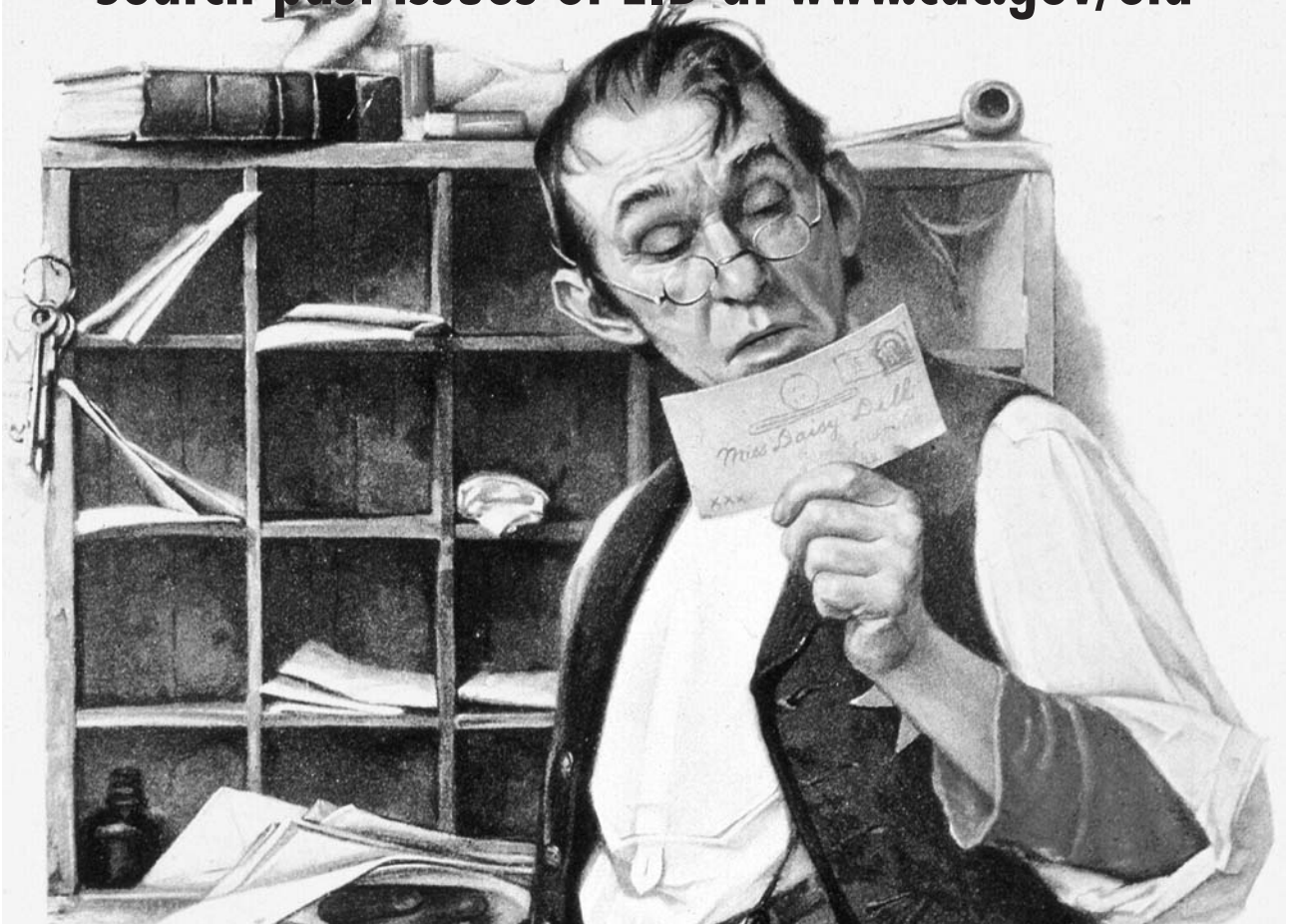
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# High Incidence of Pulmonary Tuberculosis Persists a Decade after Immigration, the Netherlands

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Suzanne Verver,† Caspar W.N. Looman,\*  
Vivian Bos,\* Martien W. Borgdorff,†  
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Incidence rates of pulmonary tuberculosis among immigrants from high incidence countries remain high for at least a decade after immigration into the Netherlands. Possible explanations are reactivation of old infections and infection transmitted after immigration. Control policies should be determined on the basis of the as-yet unknown main causes of the persistent high incidence.

We describe patterns of incidence rates of pulmonary tuberculosis in immigrants in the Netherlands according to the length of time since immigration. Insight in these patterns is needed to evaluate tuberculosis control policies that aim to reduce transmission. The Dutch control policy differs from policies in other industrialized countries: not only is obligatory screening by chest x-ray performed at the time of immigration, but immigrants are also invited for voluntary follow-up screening at 6-month intervals in the first 2 years after immigration.

## The Study

We performed a retrospective cohort analysis of all legal immigrants notified as having pulmonary tuberculosis in the Netherlands between 1996 and 2000; pulmonary tuberculosis referred to any form of active tuberculosis that involved the lungs. Patient data were obtained from the Netherlands Tuberculosis Register and included date of birth, date of arrival in the Netherlands, time of diagnosis, localization of tuberculosis, country of origin, and sex. To account for the fact that the reported time of immigration was often exactly 1, 2, 3, . . . years before diagnosis ("digit preference"), time since immigration was categorized with boundaries well apart from the preferred digits (Table).

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Data on the number of immigrants residing in the Netherlands were obtained from the Organization for Reception of Asylum Seekers (COA) and from municipal population registers (GBA) as provided by Statistics Netherlands. Person-years at risk for pulmonary tuberculosis were first calculated separately for both the COA and GBA registers. Privacy regulations prohibit matching of the two datasets. Since asylum seekers are allowed to register themselves in the GBA after 1 year of stay in the Netherlands, overlap between the two registers had to be accounted for. We assumed that the percentage of asylum seekers registered twice increased linearly from an initial 0% of asylum seekers in the COA register during the first 6 months after immigration, to 80% at 3.5 years after

Table. Incidence rate and relative risk of pulmonary tuberculosis according to time since immigration, country of origin, age, sex, and year of diagnosis for immigrants in the Netherlands, 1996–2000

	Incidence rate/100,000 person-years (cases)	Multivariate relative risk (95% CI)
Time since immigration (y)		
0.5–1.4	59 (292)	1.39 (1.14 to 1.69)
1.5–2.4	44 (169)	1.00
2.5–3.4	55 (166)	1.14 (0.91 to 1.43)
3.5–4.4	43 (118)	0.88 (0.69 to 1.11)
4.5–6.4	42 (245)	0.89 (0.72 to 1.09)
6.5–9.4	34 (247)	0.80 (0.65 to 0.98)
9.5–19.4	21 (338)	0.58 (0.48 to 0.71)
≥19.5	15 (430)	0.49 (0.40 to 0.60)
Country of origin		
Morocco	47 (334)	1.83 (1.57 to 2.14)
Somalia	379 (392)	11.30 (9.63 to 13.25)
Other Africa	69 (270)	2.14 (1.82 to 2.52)
Turkey	21 (178)	0.83 (0.69 to 1.00)
Asia	25 (419)	1.00
Suriname and Antilles	16 (194)	0.68 (0.57 to 0.81)
Latin America	19 (33)	0.76 (0.53 to 1.09)
Central and Eastern Europe	22 (100)	0.74 (0.59 to 0.93)
Other countries	5 (86)	0.21 (0.16 to 0.26)
Age (y)		
0–14	13 (78)	0.25 (0.20 to 0.32)
15–24	45 (412)	1.00 (0.88 to 1.13)
25–34	39 (661)	1.00
35–44	28 (424)	0.99 (0.87 to 1.12)
45–54	17 (185)	0.81 (0.68 to 0.97)
55–64	17 (117)	0.87 (0.71 to 1.08)
≥65	19 (128)	1.32 (1.05 to 1.64)
Sex		
Male	37 (1,291)	1.62 (1.48 to 1.78)
Female	20 (714)	1.00
Y of diagnosis		
1996	31 (413)	1.00
1997	30 (408)	0.97 (0.85 to 1.12)
1998	25 (356)	0.80 (0.70 to 0.93)
1999	28 (408)	0.87 (0.76 to 1.00)
2000	27 (421)	0.83 (0.73 to 0.96)

immigration. We recognize the arbitrariness of this assumption. Therefore, we carried out a sensitivity analysis with contrasting assumptions— asylum seekers were never versus always registered twice—to assess the consequences of the uncertainty regarding double registrations. This did not alter the conclusions (results not shown).

By the end of 2000, close to two million immigrants were residing in the Netherlands, of a total population of nearly 16 million. Among the immigrant population, 2,661 patients with pulmonary tuberculosis were identified during 1996–2000. Information about country of origin and time since immigration was missing in 3% and 13% of the study patients, respectively, and was accounted for by multiple imputation (five times) to avoid bias in the calculation of incidence rates, relative risks, and confidence intervals (1). For country of origin and time since immigration, all information presented is based on the average number of cases in the imputed datasets.

Incidence rates were only calculated for the 2,005 patients in whom tuberculosis was diagnosed more than half a year after immigration because many patients with a case diagnosed within 6 months may already have had active tuberculosis at the time of immigration. These patients should be considered prevalent rather than incident cases.

The Figure shows that incidence rates decreased after 0.5–1.4 years since immigration for immigrants from most of the countries. Subsequently, the incidence rates were mostly stable from 1.5 to 9.4 years since immigration for the countries with initial incidence rates above or around 50/100,000 (as a general rule, immigrants from countries with incidence rates above this level are eligible for screening). African immigrants, especially Somalis, had the highest incidence rates. Since few Somalis immigrated before 1991, the observed increase in incidence rates >9.4 years after immigration has wide confidence intervals. In contrast to the incidence rates for most of the countries, incidence rates for immigrants from Suriname and the Netherlands Antilles were initially low and significantly increased after an initial decrease. Average incidence rates after immigration varied from 379/100,000 in Somalis to 5/100,000 in immigrants from the category “other countries” (Table). For comparison, the current incidence rate of pulmonary tuberculosis in the indigenous Dutch population is approximately 3/100,000.

Univariate and multivariate Poisson regression were performed by using Stata (Stata Corp; College Station, TX). For each imputed dataset, all risk factors were significant in the multivariate regression. The Table provides the combined multivariate results. A clear pattern in incidence rates was not observed in the first 3.4 years after immigration, but overall the incidence rates gradually decreased as time since immigration increased. Nonetheless, compared

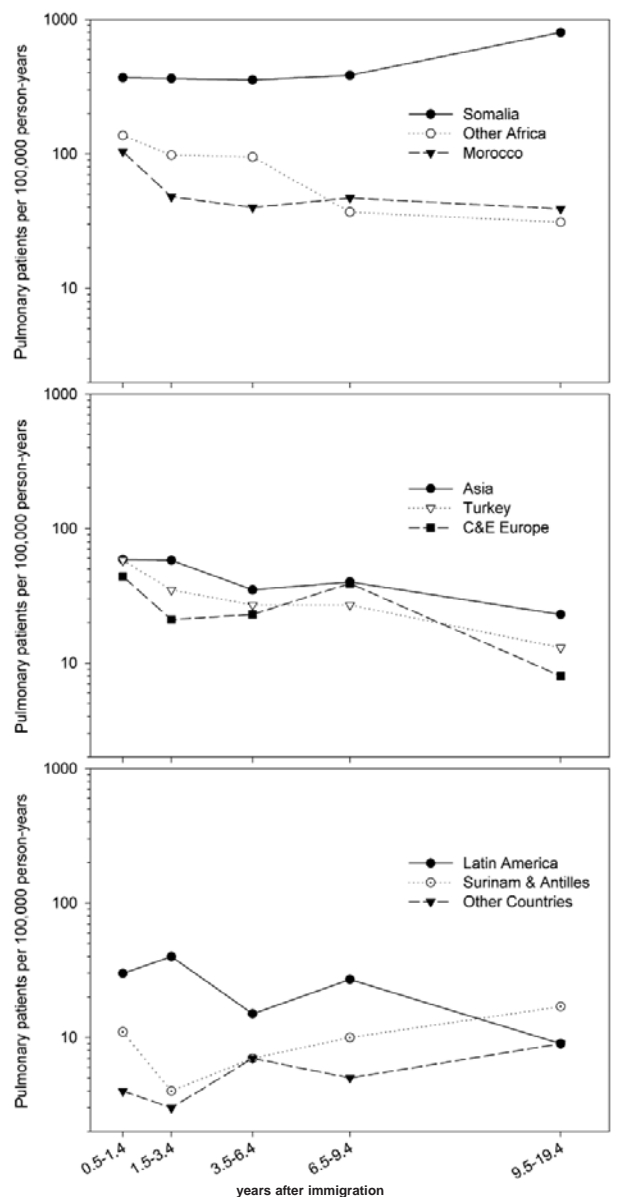


Figure. Incidence rates after immigration, according to country of origin. Central and Eastern (C&E) Europe includes Cyprus and the former Soviet Union.

to 1.5–2.4 years, the incidence rate for 9.5–19.4 years since immigration had decreased by only 42%. Fifty-eight percent of patients, including those in whom tuberculosis was detected in the first 6 months, were found more than 2.5 years after immigration to the Netherlands, and 29% were found after more than 9.5 years.

As often observed, we found considerably lower incidence rates for children than for young adults and a significantly higher rate for males than females. Except for age, the univariate incidence rate ratios were largely similar to the multivariate ratios. In univariate analysis, incidence



rate ratios in adults decreased with age, whereas in multivariate analysis the oldest age group had an increased risk. This result is due to confounding with country of origin and time since immigration: African immigrants had the highest incidence rates, but relatively few of them were older than 65 years, and they had immigrated relatively recently. Statistically significant, but small, differences in incidence rates according to year of diagnosis were observed (Table).

## Discussion

Our study shows that, in spite of a gradual decrease, the incidence rates of pulmonary tuberculosis in immigrants remain high even a decade after immigration. The persistent high incidence rates are consistent with results of previous studies (2–5). Our study combines data on all immigrant patients in whom tuberculosis was detected and all legal immigrants present in a 5-year period in a low incidence country, enabling detailed analysis with a long follow-up period.

We did not find a steep decline in incidence rates after immigration. One might anticipate such a decline, since the proportion of recently infected or reinfected persons will be higher sooner after immigration than later due to relatively low levels of transmission in the Netherlands. Recent infection is a known risk factor for developing active tuberculosis (6,7). Several explanations may account for the absence of an initial steep decline in incidence rates. First, the proportion of immigrants who were recently infected or reinfected may already have been low at the time of immigration. Next, the risk of reactivation of latent tuberculosis infection in these immigrants may have been higher than previously modeled in white nonimmigrant populations (8,9). Finally, immigrants residing in the Netherlands may have acquired new infections or reinfections, either through transmission within the Netherlands or through frequent visits to their country of origin. DNA fingerprinting data suggest that transmission within the Netherlands may indeed have occurred, although it is not the key factor; in a recent study, infections in 30% to 40% of Turkish, Moroccan, and Somali patients could be attributed to recent transmission, but 58% of all immigrant patients were not part of a cluster (10).

The Dutch screening policy consists of mandatory screening of immigrants at entry and voluntary screening in the next 2 years. Less than 50% of immigrants undergo voluntary screening in the second year (11). Screening identified 41% of the patients with a case diagnosed from 0.5 to 2.4 years after immigration. Screening may have influenced the observed incidence pattern slightly by diagnosing cases earlier than in the absence of screening. However, the average delay in detecting tuberculosis in immigrant patients who seek medical care themselves

(passive detection) in the Netherlands is <3 months (12), and several studies reported upon by Toman (13) suggest that the period in which tuberculosis is detectable by x-ray, but has not yet led to clinical symptoms (preclinical detectable phase), is <6 months. Thus the incidence pattern in the first view years after immigration would not be very different in the absence of screening. The possible influence of screening on transmission has apparently not resulted in a pronounced downward trend in incidence rates over time: they would only have remained somewhat higher without screening.

In many industrialized countries, an increasing proportion of tuberculosis patients are immigrants. Immigrants account for >50% of the incidence in the Netherlands (12). Control policies with regard to immigrant tuberculosis usually rely on chest x-ray screening and treatment of active tuberculosis. A supplemental approach, recommended by the Institute of Medicine (14), is to conduct tuberculin skin testing and to apply preventive treatment of latent infections. Whether all tuberculin skin test–positive immigrants should be treated, or only selected high-risk groups such as immigrants with radiographic evidence of inactive disease, is under debate (15). Adherence to preventive treatment is also a point to consider (15). To answer the question of why the incidence rates remain high, the relative importance of three factors needs to be established: reactivation of old infections, transmission in the host country, and infections acquired during visits to the countries of origin. These answers are essential to evaluate the cost-effectiveness of the Dutch screening policy and of alternative options, including other screening policies and use of preventive treatment.

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# Murine Typhus with Renal Involvement in Canary Islands, Spain

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Murine typhus and “murine-typhus-like” disease are reemerging infectious diseases. In Canary Islands (Spain), a rather distinct clinical pattern characterized by higher incidence of complications, especially renal damage (including acute failure and urinalysis abnormalities), is apparent and highly suggestive. It could be related to different strains of *Rickettsia typhi* or other cross-reactive species.

## The Study

Murine or endemic typhus is caused by *Rickettsia typhi*, formerly *R. mooseri* (1). Classic murine typhus is a zoonosis maintained in rats (*Rattus rattus* and *Rattus norvegicus*) and transmitted to humans through damaged skin by infected feces from the oriental rat flea (*Xenopsylla cheopis*) (2). New patterns of disease (“murine typhus-like” disease) have been described in recent years, and a new species of *Rickettsia* (*R. felis*) that causes a similar clinical picture has been identified (1–3). New modes of infection have been identified, including infection through inhalation of flea feces and transmission by different types of fleas (*Ctenophthalmis felis*) and from different reservoirs (e.g., dog, cat, and opossum).

Murine typhus occurs worldwide, particularly in warm and humid climates (1). In Spain, two seroepidemiologic surveys, in Salamanca and Madrid (Central/Western Spain), yielded seroprevalence rates of 12.8% and 68%, respectively, in the general population (4,5). However, no clinical cases have been reported. In Seville (Southwestern Spain), murine typhus is an important cause of fever of intermediate duration (6), and in Canary Islands, 10 autochthonous cases have been reported from Tenerife (7). For this reason, we include serologic testing for *R. typhi* in

the evaluation of patients with fever of intermediate duration. We describe the clinical picture of murine typhus in the Canary Islands.

Adult ( $\geq 14$  years of age) inpatients and outpatients at the Hospital Universitario Insular of Las Palmas with a serologic diagnosis of murine typhus during December 1, 2000, through July 30, 2002, were included in our study. A case was defined by an immunoglobulin (Ig) M titer  $\geq 1:40$ , or a fourfold or higher increase in IgG titers against *R. typhi* by direct immunofluorescence test in 8 weeks (bioMérieux, France), or both.

Epidemiologic, clinical, and laboratory data were collected. Antibodies against other agents that may cause a fever of intermediate duration (e.g., *Coxiella burnetii*, *R. conorii*, *Leptospira* sp., Epstein-Barr virus, and cytomegalovirus) were systematically tested. Twenty-two patients (21 men, 1 woman), with a mean age of 28 years (range 14 to 76 years), were included. Murine typhus was more frequent in summer (Figure 1). No case aggregation was observed. The geographic distribution is shown in Figure 2. All patients reported contact with animals (13 with dogs, 6 with horses, 5 with goats, 2 with cats, and 1 with camels).

The main symptoms and signs recorded are shown in Table 1. All patients had a high fever (mean 39.3°C) during a mean of 10 days (range 7 to 20 days). A light maculopapular, nonpurpuric rash, with rather centripetal distribution, was a frequent finding (68.2%). Up to one third of the patients had a dry cough. Hepatomegaly and, less frequently, splenomegaly were detected. Skin lesions suggestive of insect bites were found in 13.6% of the patients.

Four patients had a mild normocytic anemia. For most patients, leukocyte counts were normal, mild leukopenia was detected in two patients, and mild leukocytosis in four

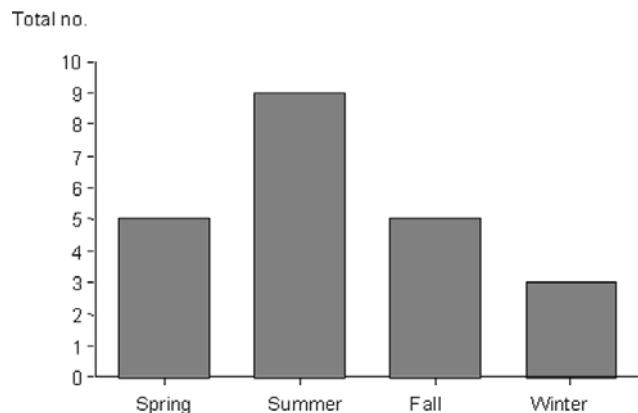


Figure 1. Distribution of cases of murine typhus by season.

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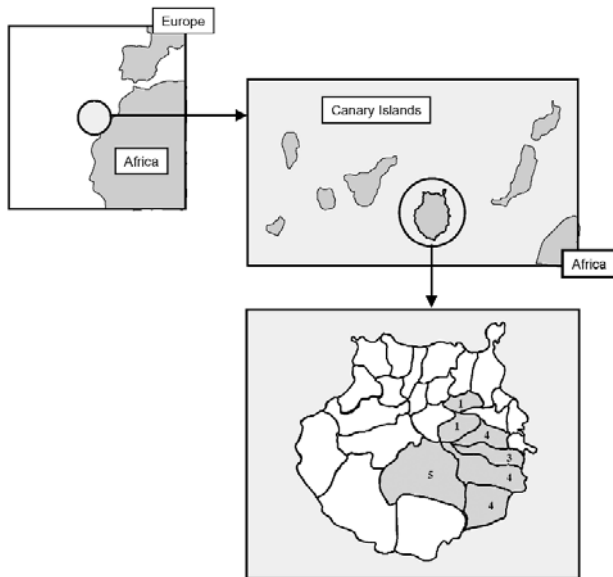


Figure 2. Distribution of cases of murine typhus by geographic area.

patients. Ten case-patients (45%) had thrombocytopenia. In most patients (89.5%), the erythrocyte sedimentation rate was high (11–83 mm/h), and the activated partial thromboplastin time (aPTT) was prolonged in six patients.

Aminotransferase elevation, usually four times above normal, was found frequently; two patients had normal values. Four patients had alanine aminotransferase values 10 times the normal value. Plasma bilirubin was normal for all patients.

In 36% of the patients, the plasma blood urea nitrogen was elevated; plasma creatinine was above normal in three cases (13%). In 19 cases (87%), alterations were found in the urinalysis. Fifteen patients had proteinuria and microhematuria with or without leukocyturia and granular casts,

with a negative nitrite reaction. In two patients, isolated proteinuria occurred, and isolated microhematuria occurred in two other patients. All of these findings resolved quickly.

Eight patients fulfilled both diagnostic criteria (IgM  $\geq 1:40$  plus seroconversion), eight patients had initial IgM elevation, and six seroconverted without IgM increase. Cross-reactivity between *R. typhi* and other microorganisms was not observed. Fifty percent of the patients had serologic evidence of past infection with *C. burnetii* (12/22) or *R. conorii* (3/22) and, in one case, of co-infection with *C. burnetii*.

Eight cases were not treated because of spontaneous recovery. The remaining patients received doxycycline (100 mg twice a day). Fever disappeared from 1 to 6 days (median 2 days).

Three patients had severe signs and symptoms. Patient 6 was admitted with acute respiratory failure, lung infiltrates, and acute renal failure (plasma creatinine 2.8 mg), microhematuria, and leukocyturia. Intravenous fluids, doxycycline, ciprofloxacin, and methylprednisolone (1 g) were administered, and the patient rapidly improved. Autoantibodies and cryoglobulins were negative. Patient 16 had a dry cough and acute renal failure (plasma creatinine 2.7 mg) and later become disoriented. A cranial contrast computed tomography scan was normal, and cerebrospinal fluid (CSF) showed mononuclear pleocytosis (90 cells/ $\mu$ L), protein 70 mg/dL, and normal glucose. Doxycycline was administered with rapid neurologic improvement. Conjunctivitis and rash appeared but waned shortly after. Finally, patient 21 had a progressive meningeal syndrome, CSF showed mononuclear pleocytosis (19 cells/ $\mu$ L), increased protein (49 mg/dL), and normal glucose levels. The patient completely recovered in 48 hours after receiving doxycycline.

Table 1. Clinical findings<sup>a</sup>

Clinical findings	Researcher and location of study						
	Dumler, TX 1991	Silpapojakul, Thailand 1992	Bernabeu, Seville 1999	Fergie, TX 2000	Whiteford, TX 2001	Gikas, Crete 2002	Canary Islands 2003
n	80	137	104	30	97	83	22
Fever	98	100	100	100	100	100	100
Headache	75	41	71	77	76	88	90
Rash	54	20	62	80	63	80	-
Arthromyalgia	46	44	77	57	40	45	45
Hepatomegaly	-	24	29	-	-	22	38
Cough	35	-	25	40	15	28	28
Diarrhea	26	5	5	40	33	11	18
Splenomegaly	-	5	24	-	-	23	14
Bite	39	0	3	3,3	34	-	13
Nausea/vomiting	48	3	23	43	45	18	13
Abdominal pain	23	10	-	60	27	11	13
Confusion	8	2	4	7	8	10	13

<sup>a</sup>Data are expressed as a percentage; TX, Texas.

Table 2. Comparative laboratory findings in patients with murine typhus

Laboratory test results	Researcher and location of study						
	Dumler, TX	Silpapojakul, Thailand	Bernabeu, Seville	Fergie, TX	Whiteford, TX	Gikas, Crete	Canary Islands
Anemia	75	-	1	57	69	25	18
Leukopenia	28	4	18	40	37	7	9
Leukocytosis	29	-	20	3	1	0	18
Neutrophilia	-	-	-	63	77	-	36
Thrombocytopenia	48	3	19	60	43	51	45
ESR elevated	-	-	59	75	81	-	89
Increased ratio prothrombin time	30	-	-	-	-	-	23
Increased ratio aPTT	-	-	-	-	-	-	27
Plasma BUN increased	27	-	-	0	3	-	36
Plasma creatinine increased	21	-	-	0	0	-	14
Hyponatremia	60	-	-	66	58	37	18
Plasma creatine kinase increased	21	-	-	-	-	42	10
Plasma LDH increased	87	-	-	-	100	82	81
Plasma AST increased	90	-	67	67	82	86	77
Plasma ALT increased	73	-	67	67	38	64	99
Plasma alkaline phosphatase increased	60	-	25	-	-	15	30
Plasma GGT increased	-	-	-	-	-	-	57.2
Hypoalbuminemia	89	-	-	46	87	82	54.5
Hypergammaglobulinemia	-	-	-	-	-	-	75.0

<sup>a</sup>Data are expressed as a percentage. TX, Texas; ESR, erythrocyte sedimentation rate; aPTT, activated partial thromboplastin time; BUN, blood urea nitrogen; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma glutamic transferase.

Fever of intermediate duration has been defined by others in Spain as fever of 7 to 28 days without localizing signs (i.e., respiratory, digestive, urinary, or neurologic), plus the absence of diagnostic clues after a complete evaluation (6). A few diseases can account for most cases of this type of fever (mainly Q fever, brucellosis, boutonneuse fever, leptospirosis, mononucleosis syndromes, and murine typhus). In our area, autochthonous cases of boutonneuse fever or brucellosis have never been reported. Diagnosis is usually based on serology, which requires time for confirmation. Therefore, in the meantime, identifying clinical data for empirical treatment is important.

In our study, the number of cases per year is 12, higher than that in other areas of Spain (6), Israel (8), or the United States (9,10), with higher rate in summer. Most patients were male. All patients had direct contact with animals as reported by others (9,10); dogs were the most frequently cited animal (9,10).

The clinical features in our study are similar to those reported by others (6,9–13) with respect to those most frequent symptoms (fever, headache, and arthromyalgia) (Table 1). The incidence of rash is similar to that reported by Bernabeu (6) and Whiteford (9) and higher than that in other series. Reports of insect bites were more frequent in our study than studies from other areas (Bernabeu [6] and Silpapojakul [11]), but more insect bites were reported from a Texas study (9).

The laboratory findings in our study are similar to findings in other studies, although its relative frequency is

variable (Table 2). The frequency of anemia varies from 1% to 69%, leukopenia from 4% to 40%, and thrombocytopenia from 3% to 60% (6,9,10,11). While 80% of the patients with Q fever in our area have a prolonged aPTT, 27% of the patients with murine typhus displayed this abnormal coagulation test. An elevation of aminotransferases in the range of viral hepatitis was common, but hyperbilirubinemia is exceptional and usually associated with alcoholism, co-infection, or glucose 6-phosphate dehydrogenase deficiency.

Nephrologic alterations had a high frequency in our study. Three patients had acute renal failure, and 87% had some abnormality in the urinalysis, mainly microhematuria. These data are in sharp contrast with the low incidence of urinary alterations found in other studies. Some broad studies (6,9,10) do not report urinary abnormalities in murine typhus, though Dumler et al. (13) reported microhematuria or proteinuria in 28% of their patients. In a study specifically focused on renal involvement in murine typhus, Shaked et al. observed urinary abnormalities in 5 of 27 patients studied (8). To the best of our knowledge, 11 cases of acute renal failure have been related to *R. typhi* (9,11,14,15).

In general, murine typhus is a mild disease. However, a number of miscellaneous complications have been described. Our severe cases accounted for 13% (one renopulmonary syndrome, one encephalitis, and one meningitis with renal failure).



## Conclusions

In summary, in the Canary Islands, incidence of murine typhus seem to be higher, patients more frequently report contact with dogs, the frequency of complicated cases is higher, and the incidence of renal involvement is higher. These data define a clinical picture of murine typhus that is somewhat different for the Canary Islands. These differences could be attributed to age (infantile versus adult series), mode of transmission or infection, or different strains of *R. typhi*. The diagnostic methodology was indirect, so cross-reaction with other rickettsiae cannot be excluded (11). Moreover, in our area, dogs are frequently parasitized by the flea of cats, a well-known vector for *R. felis* (3). More studies with direct diagnostic methods are needed to better define these differences. Finally, detecting urinary abnormalities in the setting of fever of intermediate duration, especially if associated with skin rash, thrombocytopenia, or hypertransaminasemia, in our geographic area is strongly suggestive of murine typhus.

Dr. Hernández-Cabrera was an associate professor of infectious diseases and tropical medicine at Faculty of Medicine at University of Las Palmas de Gran Canaria, Spain. His research interests focus on rickettsial diseases and other causes of fever of intermediate duration.

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# *Anaplasma phagocytophilum*, *Babesia microti*, and *Borrelia burgdorferi* in *Ixodes scapularis*, Southern Coastal Maine

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Charles Lubelczyk,\* Peter W. Rand,\*  
and Robert P. Smith\*

*Ixodes scapularis* (deer ticks) from Maine were tested for multiple infections by polymerase chain reaction and immunofluorescence. In 1995, 29.5%, 9.5%, and 1.9% of deer ticks were infected with *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti*, respectively. In 1996 and 1997, the number of *A. phagocytophilum*-infected ticks markedly declined. In 1995 through 1996, 4 (1.3%) of 301 were co-infected.

Throughout its range in the eastern and upper midwestern United States, *Ixodes scapularis* (*Ixodes dammini*) (deer tick) is the vector of *Borrelia burgdorferi*, the causative agent of Lyme disease. In recent decades, it has been associated with several other pathogens, including bacteria, viruses, and protozoa, a guild of pathogens similar to that seen in the related tick *Ixodes ricinus* in Europe (1).

*I. scapularis* was determined to be the vector of the intraerythrocytic protozoan *Babesia microti* on Nantucket Island, Massachusetts in 1979 (2). Human granulocytic ehrlichiosis (HGE) was first described in 1994 in patients from Wisconsin and Minnesota (3). *I. scapularis* was determined to be a competent vector of the obligate intracellular bacteria that cause HGE, and field-derived ticks from Massachusetts were found to be co-infected with the HGE agent and *B. burgdorferi* (4). The agent of HGE, previously referred to as *Ehrlichia phagocytophila*, has recently been reclassified as *Anaplasma phagocytophilum* (5).

Rodents and birds have been demonstrated to be reservoirs of the Lyme disease spirochete in areas of Maine

where the tick is established (6). This study sought to determine if *I. scapularis* at the northern edge of its range was infected with *A. phagocytophilum* and *Ba. microti*, in addition to *B. burgdorferi*.

## The Study

*I. scapularis* nymphs and adult females that had partially fed on a variety of hosts were collected in 1995 through 1997 from coastal areas in Maine, from York to Hancock counties, where the tick is established and Lyme disease is endemic (Figure A). Ticks removed from pets and humans were submitted to our laboratory for species confirmation. Nymphs were also removed from white-footed mice and eastern chipmunks live-trapped on established research grids in the town of Wells and from Norway rats trapped on an offshore island. Mammal trapping procedures were approved by the Maine Medical Center Institutional Animal Care and Use Committee. One *I. scapularis* female was removed from a nontranquilized, live, white-tailed deer that had become accustomed to humans on Monhegan Island. All ticks were transported alive to the laboratory.

Ticks were dissected on sterile glass slides in a drop of 10 mmol Tris-HCl, 1 mmol EDTA pH 8 (TE). Salivary glands were isolated, and one gland from each tick was stained by the Feulgen reaction for microscopic examination for inclusions (7); the other gland was prepared for DNA extraction. A smear of tick midgut was prepared for fluorescent microscopic examination for spirochetes as described previously (6).

All polymerase chain reaction (PCR) tests were performed on salivary glands from individual ticks except for 14 instances in 1995 when salivary glands from several ticks collected from an individual host were pooled for PCR analysis. For statistical purposes, when a PCR product was obtained from a pool of salivary glands from multiple ticks, only one tick in the pool was assumed to be infected.

Salivary glands were stored at  $-20^{\circ}\text{C}$  in 50  $\mu\text{L}$  of TE buffer until DNA extraction. DNA was isolated using a standard phenol/chloroform extraction procedure (8) or by using the IsoQuick kit (ORCA Research, Bothell, WA) according to the manufacturer's protocol and placed in 20  $\mu\text{L}$  of TE buffer. Sterile aerosol-barrier tips were used during all procedures. DNA isolation and PCR reactions were performed in separate laboratories. Positive and negative controls were included in each PCR reaction.

*Babesia* was detected by amplifying a 437-bp portion of the eukaryotic 18S rRNA gene by PCR using primer pair PiroA/PiroB (9). Components were denatured at  $94^{\circ}\text{C}$  for 45 sec, annealed at  $60^{\circ}\text{C}$  for 45 sec, and extended at

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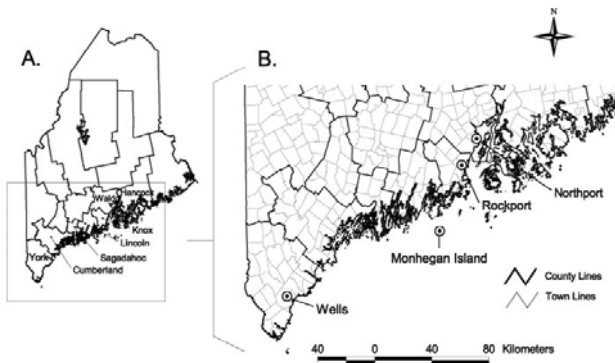


Figure. A. Counties in southern Maine where ticks were collected. B. Four towns where ticks infected with *Anaplasma phagocytophilum* or *Babesia microti* were found.

72°C for 2 min, for a total of 40 cycles. Samples were separated by electrophoresis on a 1% Sea Plaque agarose gel containing ethidium bromide and 40 mmol Tris-acetate 1 mmol EDTA pH 8.3 buffer.

*Anaplasma* was identified by the amplification of 16S rDNA by PCR. The primer pair consisting of GE9 (3) and Ehr747 (10) was used to generate an 849-bp fragment. The thermal cycling profile used was the same as for *Babesia*.

Amplified products were excised from the gels, treated with Beta-agarase (Sigma, St. Louis, MO), cycle-sequenced using dye-labeled dideoxy terminators (Applied Biosystems Big Dye Reaction Kit, Foster City, CA) and purified by using Centri-Sep columns (Princeton Separations, Adelphia, NJ). Samples were electrophoresed on a 6% polyacrylamide stretch gel using an ABI 373A DNA sequencer. DNA sequences were compared with previously published sequences for species identification, using the Sequence Navigator program by Applied Biosystems.

From 1995 to 1997, PCR was performed on salivary glands from 223 *I. scapularis* nymphs and 171 females. Nymphs comprised 44% of ticks tested the first year of the study and 61% in both of the later years. The Table presents the prevalence of infection with *A. phagocytophilum*, *Ba. microti*, and *B. burgdorferi* in *I. scapularis* studied each year.

Four of the positive PCR results were obtained from pooled glands. Assuming only one gland in each pool was

positive, a total of six nymphs (possible range 6–12) and five female *I. scapularis* (possible range 5–7) were infected with *A. phagocytophilum*. *Ba. microti* was found in two nymphs and one female tick. Nine of the infected ticks were collected in the town of Wells in York County, three were from Monhegan Island in Lincoln County, and one each was from the towns of Rockport in Knox County and Northport in Waldo County (Figure B). Four nymphs were infected with two organisms (Table). All of the co-infected ticks were from the town of Wells in York County.

*Babesia* spp. piroplasms were microscopically visualized by the Feulgen reaction in salivary acini from 21 ticks. Two glands positive for *Babesia* spp. by visual inspection had PCR product that matched sequences for *Ba. microti*; the remaining 19 (90.5%) of 21 samples matched sequences for *Ba. odocoilei*, a parasite of deer not known to cause human illness (9). Two (18%) of 11 feulgen-stained glands from ticks determined to be positive for *A. phagocytophilum* by PCR were considered positive by visual inspection of the other gland. All amplification product from the *A. phagocytophilum*-positive ticks had 99% homology (848/849 bp) with sequences of *E. phagocytophila*-human agent of Chen et al. (GenBank accession no. U02521) (3).

## Conclusions

Multiple studies conducted in hyperendemic areas of Lyme disease have reported *A. phagocytophilum* and *Ba. microti* in field-collected *I. scapularis* (4,7,10–13). Schwartz et al. reported an increase in the percent of adult deer ticks infected with the agent of HGE in Westchester County, New York from 32% of ticks collected in 1984 and tested retrospectively, to 53% in 1995 (11). In a 2-year study in Connecticut, 12.5% of adult ticks in 1996 and 19% in 1997 were infected with *A. phagocytophilum* (12). The current study showed a decrease in the percent of infected ticks collected from the same geographic areas for a 3-year period. *A. phagocytophilum* infection rates declined from 9.5% in 1995 to 0.5% and 0% in subsequent years. The percent of ticks infected with *B. burgdorferi* remained relatively constant for the 3-year period (Table).

*Ba. microti* infection rates based on DNA sequences of the organism have been reported from 5% of adult ticks

Table. Prevalence of *Anaplasma phagocytophilum*, *Babesia microti*, and *Borrelia burgdorferi* in *Ixodes scapularis*, Maine, 1995–1997

Y	n	No. (%)				
		<i>A. phagocytophilum</i>	<i>Ba. microti</i>	<i>B. burgdorferi</i>	<i>B. burgdorferi</i> and <i>A. phagocytophilum</i>	<i>B. burgdorferi</i> and <i>Ba. microti</i>
1995	105	10 (9.5) <sup>ab</sup>	2 (1.9) <sup>a</sup>	31 (29.5) <sup>a</sup>	2 (1.9)	1 (1.0)
1996	196	1 (0.5)	1 (0.5) <sup>a</sup>	35 (17.9) <sup>a</sup>	0	1 (0.5)
1997	93	0	0	22 (23.7)	0	0

<sup>a</sup>Total includes co-infected ticks.

<sup>b</sup>Four pools of salivary glands from 2–3 ticks from the same host tested positive. This table presents data assuming only one tick from each pool was infected.



tested in New Jersey (13) to 9% of adult ticks on Nantucket Island in Massachusetts (4). In 1995, 1.9% of ticks tested in this study were positive for *Ba. microti*; the percent infected dropped in subsequent years to 0.5% and 0%. This low prevalence of *Ba. microti* infection in Maine ticks is not unexpected. Mather et al. reported that *Ba. microti* was found only in areas of Rhode Island where tick abundance reached >20 nymphs per hour of flagging (14). In our study, the three ticks infected with *Ba. microti* were collected in the town of Wells in coastal York County where tick density is the highest in the state (unpub. data). Although enzootic *Ba. microti* maintained by *Ixodes angustus* or other nidicolous ticks may be widespread in Maine, *I. scapularis* density high enough to support zoonotic transmission of *Ba. microti* may only occur in a few foci (15).

That the prevalence of infection of ticks with *B. burgdorferi* during this 3-year study remained fairly constant while that of *A. phagocytophilum* showed greater variation is of interest. Other researchers have shown that white-footed mice remain reservoir competent for *A. phagocytophilum* for short periods of time (16) and that transmission of multiple organisms may have a different dynamic than that of single pathogens (17). Few studies have followed the natural infection of tick hosts with multiple organisms over time. This study indicates that the prevalence of these emerging pathogens may not be as stable from year to year as is the rodent-*I. scapularis*-*B. burgdorferi* cycle.

This study provides evidence of the potential for human exposure to multiple tick-borne pathogens in southern coastal Maine and that the risk for exposure to *A. phagocytophilum* may vary considerably from year to year.

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# Dengue Fever Outbreak in a Recreation Club, Dhaka, Bangladesh

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An outbreak of dengue fever occurred among employees of a recreation club in Bangladesh. Occupational transmission was characterized by a 12% attack rate, no dengue among family contacts, and *Aedes* vectors in club areas. Early recognition of the outbreak likely limited its impact.

Large outbreaks of dengue fever are rarely reported from occupational or institutional settings (1), probably because a small proportion of *Aedes* mosquitoes are infected with dengue viruses (2), and in dengue-endemic areas, many adults are immune. Dengue has recently reemerged in Bangladesh; in contrast with the situation in countries where dengue has long been endemic, adults appear to become ill with dengue more often than children (3).

We investigated an outbreak of dengue fever among employees of a Recreation Club for expatriates in Dhaka. The 636-member club, which occupied 92,820 sq ft within a residential area, had 107 employees. Initial cases were evaluated by an embassy physician in early October 2001. Club management requested our investigation to define the magnitude of the outbreak and recommend prevention and control strategies.

## The Study

We defined a case of dengue as a febrile illness lasting  $\geq 3$  days during September or October, 2001, with confirmation of dengue infection by presence of antibodies in sera consistent with dengue infection or with presence of dengue viruses in acute-phase sera, detected by reverse transcriptase–polymerase chain reaction (RT-PCR). Cases were identified through occupational absentee logs and through results of initial laboratory testing of acute-phase sera (as ordered by physicians).

Two batches of serum specimens were tested: 18 serum specimens were collected from ill persons by their physicians, and all consenting employees were asked to provide serum specimens 1 month after the outbreak (specimens were collected on November 21, 25, and 26, 2001). Among acute-phase sera, specimens from five patients, collected

during the first 5 days of illness, were evaluated for dengue viruses by RT-PCR for serotype-specific dengue viral RNA (4).

Acute- and convalescent-phase sera were tested for immunoglobulin (Ig) G and IgM dengue antibodies through capture enzyme-linked immunosorbent assay (5,6) (MACELISA). Specimens with  $\geq 40$  units of IgG or IgM antibodies were considered positive for dengue infection. Ratios of IgM to IgG antibodies of  $< 1.8$  were considered indicative of secondary exposure (i.e., previous exposure to dengue virus), and a ratio of  $\geq 1.8$  was considered suggestive of primary (first-time) exposure (5).

Written informed consent was taken from each study participant. This study was approved by the ethical review committee of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Interviews with consenting employees were from November 21 through 26, 2002; standardized questionnaires collected sociodemographic information, recent illnesses, febrile illnesses among family members, behaviors and activities, severity of illness, health-seeking behavior, and medications. Detailed information was collected about activities in and around the club. Weight and height were measured; body mass index  $< 20$  kg/m<sup>2</sup> was defined as underweight (7). Data were entered into FoxPro, Version 2.6 (Microsoft Corporation, Redmond, WA) and analyzed using SPSS, Version 10.0 (SPSS Inc., Chicago, IL).

A larval survey was conducted on October 20, 2001. All objects containing water (wet containers) were noted, and water from each was sampled and investigated for presence of larvae (larvae were reared to adult stage for species identification).

One hundred (94%) of 107 employees consented to participate. Dengue fever was confirmed in 13 (12%) of 107 employees, including 12 employees who experienced illness onset within a 10-day period in October (Table 1). One case occurred 10 days earlier (13% attack rate). Twelve (92%) case-employees were male. No severe cases of dengue hemorrhagic fever occurred according to World Health Organization criteria (8), but insufficient data were available to rule out grades 1 and 2. One employee was hospitalized; none died. Eleven other employees had febrile illnesses of  $\geq 3$  days duration in September or October (Figure); however, their dengue serologic assays were negative.

Ten (77%) participants had dengue antibodies in convalescent-phase sera. Samples from three participants had antibodies present in acute-phase sera only. One patient had dengue virus detected by RT-PCR with PCR pattern consistent with dengue serotype 3 (den-3).

Ratios of IgM to IgG suggested first-time infection among seven (54%) participants and secondary infection in six participants. In addition to the 13 cases of dengue, sam-

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Table 1. Characteristics of 13 dengue fever patients<sup>a,b</sup>

Case no.	Age	Sex	MACELISA (peak outbreak period; Oct 21, 2001) <sup>c</sup>	PCR (Oct 17, 2001; with fever within the past 5 days)	MACELISA (Nov 21–26, 2001)	Onset of illness	Occupation
1	26	Male	Primary	-	Primary	10/01/01	Assistant security
2	35	Male	Secondary	-	Secondary	10/10/01	Security guard
3	40	Male	Primary	Negative	Negative	10/11/01	Security guard
4	26	Male	Secondary	-	Secondary	10/11/01	Gardener
5	24	Male	Primary	Negative	Negative	10/12/01	Receptionist
6	22	Male	Secondary	Negative	Secondary	10/13/01	Tennis ball boy
7	27	Male	Negative	Negative	Secondary	10/14/01	Waiter
8	33	Male	Primary	-	Primary	10/14/01	Cook
9	30	Male	Primary	-	Negative	10/15/01	Baker
10	38	Male	-	-	Secondary	10/15/01	Laundry staff
11	38	Female	Secondary	-	Secondary	10/16/01	Laundry staff
12	38	Male	Negative	Den-3	Primary	10/17/01	Confectioner
13	34	Male	-	-	Primary	10/19/01	Gardener

<sup>a</sup>Serologic survey on Nov 21–26 indicated two more employees, not included in this table, who had dengue antibodies (one primary and one secondary pattern). They did not have symptoms; thus, they were not included as case-employees.

<sup>b</sup>ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

<sup>c</sup>Fever duration > 3 days and serologically confirmed. Those who reported in the table were only for those continuously worked from October 17 to November 2001 (4 employees were no longer working at the club and not available on Nov 21–26, 2001; their MACELISA results on October 17 included one specimen positive for primary infection and the others negative).

ples from two employees had dengue antibodies detected during the November serosurvey; the employees had not been ill. One had evidence of primary infection and one of secondary infection on the basis of the IgM/IgG ratio.

In addition to fever in all 13 dengue-positive case-employees (as required by the case definition), 11 (85%) had headache, 5 (39%) had myalgias, and 3 (23%) reported gum bleeding. Nine (69%) patients sought care from clinic or hospital; four persons with dengue did not seek care and reported using medication to alleviate symptoms.

Among 308 family members residing with the 100 employees, 21 (7%) family members were reported to be ill with fever during September and October. The rate (14%) of febrile illnesses among family members was significantly higher among 11 employees with febrile illness with negative dengue assays than among the 13 dengue patients (2%;  $p = 0.04$ ; Table 2).

The first three dengue cases occurred among security guards. They spent most working hours on the perimeter of the club, particularly around the east and west sides. Overall 3 (23%) of 13 security guards were cases compared with 10 (11%) of 87 other participating employees. Three case-patients (gardener, receptionist, and tennis ball boy) shared significant time, i.e., > 1 hour, with the first three case-patients around the main entrance, as well as within the canteen, west side and laundry room, and changing room areas. Subsequent case-patients represented a wide variety of occupations, and these employees spent time in a variety of locations around the club. The staff did not have living quarters at the club.

Epidemic dengue did not occur among club members. While surveillance was not systematically conducted, 2

(0.3%) of 636 members were known to have had dengue fever during October.

We compared data from case-employees with 76 other employees (noncase-employees) who did not have febrile illnesses during September or October. Age distribution was similar for case-employees (mean 32 years) and noncase-employees (mean 36 years). Differences in sex, duration of employment, body mass index, working hours, time spent indoors or outdoors, medications, or smoking were not significant.

Case-employees (100%) were more likely than noncase-employees (83%) to spend any time within the canteen area during break time (Kendall's  $\tau$ ,  $p < 0.01$ ). Case-employees (62%) were also more likely than noncase-employees (33%) to come to the club by walking (odds ratio = 3.3; 95% confidence interval 1.0 to 11.0;  $p < 0.05$ ). Mosquito repellent use was associated with a slightly reduced likelihood of dengue infection (0% in case-employees and 9% in noncase-employees; Kendall's  $\tau$ ,  $p < 0.05$ ).

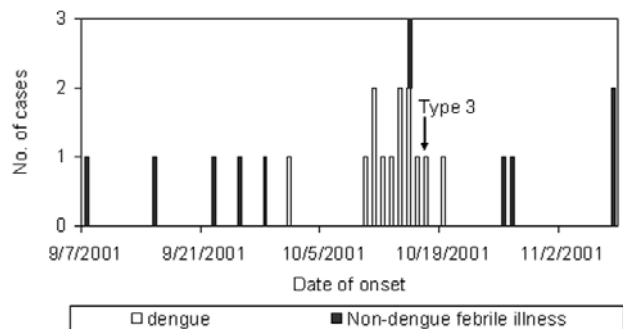


Figure. Dates of onset for cases of dengue fever and other febrile illnesses among employees of the expatriate recreation club.

Table 2. Illness reported among employees and family members during the outbreak period, September–October 2001

Employee categories	Family members with history of febrile illnesses during outbreak period	Dengue laboratory test confirmation done for febrile illnesses among family members
13 employees with confirmed dengue	2.0% (1/51) <sup>a</sup>	No laboratory confirmation done
76 employees who did not have dengue and did not have a febrile illness during outbreak period	6.5% (14/215) <sup>a</sup>	6 febrile patients had laboratory tests and 2 were serologically confirmed as having dengue fever at commercial pathology laboratories
11 employees who did not have dengue and who had a febrile illness during outbreak period	14.3% (6/42) <sup>b</sup>	No laboratory confirmation done
Total employees = 100	6.8% (21/308) <sup>a</sup>	

<sup>a</sup>Number of febrile illnesses/number of family members.  
<sup>b</sup>p = 0.04 when compared with the percentage of febrile illness among family members of case-patients.

A total of 23 larvae-positive containers were found among 34 wet containers (container index = 68) (Table 3); 364 larvae (103 *Ae. aegypti* and 261 *Ae. albopictus*) were identified in stagnant water covering surface lids of 20 metal drums used for security (to block traffic) at the west perimeter.

## Conclusions

Intense focal transmission of dengue viruses occurred within an occupational setting in a community experiencing endemic dengue. Focal intensity is highlighted by a 12% attack rate among employees for a 2-week period, compared with no known cases of dengue among nuclear family members. In other Asian countries where dengue is established and where multiple serotypes circulate, outbreaks of dengue fever in occupational settings are uncommon since adults are usually immune; disease is highest in children (8). Other febrile illnesses were occurring simultaneously among employees; these illnesses were probably caused by another communicable disease, as suggested by a higher attack rate of febrile illnesses among family members of febrile employees who did not have dengue.

Dengue viruses are transmitted by infected mosquitoes during feeding, which may occur several times a day, for a 1- to 4-week lifetime (9). The larval survey showed that *Aedes* mosquitoes were present in working areas. High concentrations of *Aedes* larvae in water on security drum surfaces may have provided a mechanism for this outbreak. The first three cases were among security guards who were often in close proximity with the security drums.

The guards also spent time inside the club (eating, praying, changing clothes, and taking breaks). The security guards may have been exposed to dengue while on patrol outside of the club, and once infected, transmitted dengue viruses to adult *Aedes* mosquitoes feeding on them while they spent time inside the club. Mosquitoes, thus infected, were able to quickly infect other employees working or resting within the club, perhaps within the staff canteen, resulting in a burst of illnesses. The end of intense transmission coincided with recognition of the outbreak, aggressive use of insecticides, and removal of breeding sites.

The outbreak likely resulted from conditions which promoted rapid transmission of dengue viruses, such as high vector density and many susceptible (nonimmune) people within close quarters. Primary infection among seven cases supports the notion that dengue has recently emerged in Bangladesh. No club members were case-patients, reflecting the importance of duration of exposure in risk for transmission.

Early recognition of the outbreak may have helped limit its impact (10). Institutional or systematic monitoring of suspected cases, i.e., surveillance, supported by prompt laboratory confirmation, may help to contain such outbreaks. Integrating and targeting vector control as soon as a cluster of cases is detected can suppress transmission and minimize numbers of cases.

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Table 3. Survey of wet containers for *Aedes* larvae within and outside of the club<sup>a</sup>

Place	Container type	No. of wet containers	No. of positive containers	No. of larvae	Mosquito species		
					<i>Aedes aegypti</i>	<i>Ae. albopictus</i>	Others
Outside of premise boundary	Metallic drum cover	26	20	364	103	261	0
Outside of club building	Plastic glass	1	1	19	0	19	0
	Manhole cover	6	1	4	4	0	0
Inside of club building	No wet container found	0	0	0	0	0	0
Rooftop of club building	Stagnant water on rooftop floor	1	1	5	5	0	0
Total		34	23 <sup>a</sup>	392	112	280	0

<sup>a</sup>Overall container index (CI) was 68 (23/34 x 100).

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It is difficult  
to get the news from poems  
yet men die miserably every day  
for lack  
of what is found there.

“Asphodel, That Greeny Flower” by William Carlos Williams, a physician and poet

## Threat of the Spores

Beware the threat of the spores,  
Bad news parcels bide their time.  
They lurk to enter the unsuspecting  
And unleash dormant evil that multiplies.

The twin rings of code within are culprits  
Spawning the Unholy Trinity of toxins.  
Inject to bleed, swell, and destroy,  
While the “protective” antigen does anything but.

Skin lesions black as the namesake coal,  
Pale in comparison to the inhaled disease.  
Finely milled, known simply as Ames strain  
Artificial and abhorrent agents of terror.

“Alpha” bacteria,  
Germs of the germ theory.  
Origin of the famous postulates,  
Crafty bugs made cruel by man.

Brought home the nesting-doll syndrome,  
Envelopes, spores, bacilli, plasmids.  
With it the fear and the frenzy  
To cure what we create.

**Setu K. Vora**

**ANOTHER DIMENSION.** Submit thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to invoke compassion for human suffering and to expand the science reader’s literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

## *Mycobacterium tuberculosis* Complex Drug Resistance in Italy

**To the Editor:** The reemergence of tuberculosis (TB) as a global health problem over the past 2 decades, accompanied by increased drug resistance, which represents a serious problem both in terms of TB control and clinical management (1), prompted Western European countries to develop comprehensive national surveillance systems to monitor trends in TB drug resistance. Moreover, the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) launched the Global Project on Anti-Tuberculosis Drug Resistance Surveillance to measure the prevalence of drug resistance by using standardized methods and assess its correlation with indicators of TB control (2,3). Since comprehensive data on resistance to firstline drugs were not available in Italy, a network of 20 regional laboratories was established to participate in this project. The Department of Bacteriology and Medical Mycology of Istituto Superiore di Sanità in Rome and the Mycobacteriology Unit of Istituto Villa Marelli in Milan (appointed respectively as Supranational Reference Laboratory

and National Reference Laboratory) supervised and controlled the network of regional laboratories. The combination of reference laboratories in the network and associated clinical units, which covered 30% of definite cases reported each year (4), was known as SMIRA (Italian Multicentre Study on Resistance to Anti-Tuberculosis Drugs). The WHO/IUATLD coordinating center in Ottawa, Canada, provided a batch of 20 *Mycobacterium tuberculosis* strains to set up proficiency testing to check drug susceptibility procedures in all SMIRA laboratories (5). We summarize the nature and extent of TB drug resistance in Italy between 1998 and 2001.

Isolates from all consecutive, definite cases diagnosed in TB units during 1998 through 2001 were included. When a patient's previous treatment status was unknown or dubious, the case was excluded. Resistant cases from patients with and without history of previous treatment were stratified by the following categories: any resistance, monoresistance, resistance to both isoniazid and rifampicin (known as rifampin in the United States), or resistance to three or more drugs. Confidence intervals were also calculated. Participating laboratories were allowed to use the WHO-recommended drug susceptibility method with which they were most familiar: absolute concentration method, resistance ratio method, proportion method

and its variants, or BACTEC 460 radiometric method (Becton Dickinson, Towson, MD) (6,7). Among the laboratories reporting results by the proportion method, the majority used Löwenstein-Jensen medium while others used liquid nonradiometric media (8). Each of the 20 *M. tuberculosis* strains was tested against firstline drugs by the Italian Reference Laboratories in Rome and Milan and classified as resistant or susceptible. Results were compared to the standard criterion, represented by the judicial results of the WHO/IUATLD Global Network of Supranational Laboratories (9). Each network laboratory was validated for each firstline drug when no more than two results were different from the standard criterion.

The prevalence of drug resistance detected during the period 1998–2001 is summarized in the Table. Among previously untreated cases, the prevalence of resistance to isoniazid, rifampicin, ethambutol, and streptomycin was 3.5%, 0.8%, 0.5%, and 4.3%, respectively, while prevalence of multidrug resistance (resistance to at least isoniazid and rifampicin) and polyresistance (resistance to two or more drugs, but not both isoniazid and rifampicin) was 1.1% and 2.4%, respectively. No difference was found by stratifying prevalence data by age, sex, or HIV status. In isolates from patients with previous treatment, drug resistance was found to be almost four

Table. Pattern of drug resistance among strains from tuberculosis patients with and without a history of treatment, Italy 1998–2001<sup>a</sup>

Tested MTB strains	No history of previous treatment			History of previous treatment		
	No.	%	95% CI	No.	%	95% CI
Total tested	2,117	100	–	322	100	–
Fully sensitive	1,847	87.2	85.8 to 88.6	155	48.1	42.7 to 53.6
Any drug	270	12.7	11.4 to 14.2	167	51.8	46.4 to 57.3
INH	75	3.5	2.8 to 4.4	17	5.3	3.2 to 8.2
RMP	17	0.8	0.5 to 1.3	14	4.3	2.5 to 7.0
EMB	10	0.5	0.2 to 0.8	1	0.3	0.02 to 1.5
SM	93	4.3	3.6 to 5.3	14	4.3	2.5 to 7.0
Resistant to both INH and RMP	8	0.40	0.8 to 0.7	24	7.5	4.9 to 10.7
Resistant to INH, RMP, EMB	2	0.10	0.01 to 0.3	19	6.0	3.7 to 8.9
Resistant to INH, RMP, SM	6	0.30	0.1 to 0.6	23	7.1	4.7 to 10.4
Resistant to INH, RMP, EMB, SM	7	0.30	0.1 to 0.6	32	9.9	7.0 to 13.5

<sup>a</sup>MTB, *Mycobacterium tuberculosis* complex; CI, confidence interval; INH, isoniazid; RMP, rifampicin; EMB, ethambutol; SM, streptomycin.

times higher than in those from patients with no history of treatment. However, the prevalence of monoresistant strains was low (5.3%, 4.3%, 0.3%, and 4.3% for isoniazid, rifampicin, ethambutol, and streptomycin, respectively) compared with the prevalence of multidrug-resistant strains whose rate reached a peak of 30.4%.

Drug-resistant TB in countries with good national control programs, such as in Western Europe, is not commonly a major health problem, although increasing immigration prompts public health authorities to maintain vigilant surveillance systems. The results of our study indicate that throughout Italy, prevalence of resistance to firstline drugs and multidrug resistance among isolates from new cases was consistently low over the 4-year survey period. Prevalence of multidrug resistance among isolates from previously treated patients was high, although a downward trend could be demonstrated during the last 2 years. Since almost 2 out of 10 isolates resistant to rifampicin were multidrug resistant, using rapid molecular methods to identify rifampicin resistance in questionable cases appears cost-effective to facilitate early detection and control of multidrug-resistant TB (10). Resistance to isoniazid is associated with immigration from countries where isoniazid was used extensively in the past. This information is a useful tool for clinicians, as isoniazid resistance may be suspected early in the disease and properly treated. Finally, the finding of substantial multidrug resistance among isolates from previously treated patients, combined with the evidence that immigrants from areas where isoniazid resistance is endemic contribute substantially to the number of new TB cases in Italy every year, strongly suggests that public health action is needed to improve treatment outcomes.

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## Mollaret-like Cells in Patients with West Nile Virus Infection

**To the Editor:** We have read with interest many of the articles concerning West Nile virus (WNV) published in the July 2003 issue of *Emerging Infectious Diseases*. Last summer Ohio was one of the leading states with WNV infection in humans. Consequently, requests for tests for this pathogen have increased. Unfortunately, the turnaround time for testing these specimens may be delayed because of shipping difficulties, the limited number of laboratories that can perform these assays, and an increase in requests at testing facilities.

Cytologic examination of cerebrospinal fluid (CSF) from patients with WNV has not been studied.

Although cytologic examination of CSF from patients with encephalitis is likely nonspecific, it may provide supportive information of the suspected disease process, and is useful for excluding other conditions, such as neoplasia. Of the 22 patients that were hospitalized at our institution last year with WNV meningoencephalitis, documented by serologic tests and/or reverse transcription-polymerase chain reaction, CSF of 4 of these patients was submitted for cytologic examination. Of these 4, 3 had a sufficient number of cells in the CSF specimen (47, 213, and 495 cell/ $\mu$ L) to afford cytologic examination, whereas one had a paucicellular CSF, with only 2 white blood cells/ $\mu$ L. The cytologic features from the 3 patients,  $>10$  cells/ $\mu$ L consistently demonstrated a mixture of lymphocytes at various stages of activation and occasional large monocytic-like cells with cerebriform nuclei reminiscent of the Mollaret cells described in CSF of patients with recurrent meningitis (Figure).

Mollaret described cells with enlarged nuclei and cerebriform nuclear contours in CSF of patients with recurrent, aseptic meningitis (1).

Although he believed these were of endothelial origin, immunohistochemical studies have subsequently shown that they are monocytes (2). This type of meningitis, now commonly known as Mollaret meningitis, has been associated with herpes simplex virus encephalitis, but the definitive cause of all cases remains unclear (3).

One of the patients infected with WNV meningoencephalitis who had Mollaret-like cells in CSF died. Postmortem neuropathologic examination showed an extensive perivascular lymphocytic infiltrate which contained mononuclear cells consistent with the Mollaret-like cells in CSF. These mononuclear cells were stained with an immunohistochemical stain directed against the CD68 antigen, which supports a monocytic origin (4). Further studies are needed to delineate the consistency of Mollaret-like cells in CSF of patients with WNV meningoencephalitis. Finding Mollaret-like cells admixed with activated lymphocytes may be a useful, readily-available test that provides supportive evidence of viral encephalitis in the appropriate clinical setting, until more definitive tests are available.

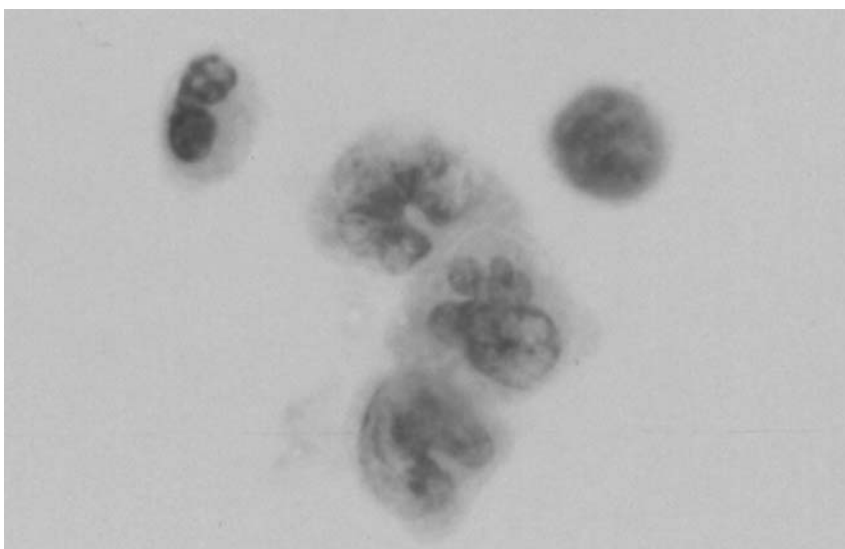


Figure. Three Mollaret-like cells are present (center), with a neutrophil (upper left) and a lymphocyte (upper right) in cerebrospinal fluid from a patient with West Nile virus encephalitis, confirmed by reverse transcription-polymerase chain reaction and serologic testing (Papanicolaou stain; magnification  $\times 500$ ).

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## Newly Isolated *Vibrio cholerae* Non-O1, Non-O139 Phages

**To the Editor:** The epidemic cholera caused by *Vibrio cholerae* O1 appeared in Latin America in 1991 after a 100-year absence. Following its explosive appearance in Peru, travelers on the Amazon River brought cholera to Brazil by April 1991. It spread southward along the Atlantic Coast of Brazil, reaching Rio de Janeiro in February 1993.

Phage typing is a useful tool for studying the source or origin of *V. cholerae* for epidemiologic importance. Because of limitations of the Basu and Mukerjee scheme, a new



phage-typing scheme for *V. cholerae* O1 was developed at the National Institute of Cholera and Enteric Diseases, India (1–3). During the course of a comprehensive study on the phage typing of *V. cholerae* O1, most strains isolated in Brazil were found to be sensitive with a set of 10 El Tor phages (ATCC 51352-B1–B10) (4). This finding prompted us to explore or ascertain the natural habitat of *V. cholerae* and cholera phages, if any, in an environmental reservoir in Brazil, particularly in Rio de Janeiro.

Samples were collected from selected points where sewage water connected with the main canal receives domestic and industrial effluents from the city on its way to the Atlantic Ocean. Two major environmental parameters, temperature and pH, were recorded at each station during collection. Samples were processed for phage isolation according to the procedure adopted for *V. cholerae* phages (5,6). Portions of the samples were also analyzed for enteric pathogens. A total of 32 sewage samples were collected in 8 months; 12 were isolated from Rio de Janeiro, 10 from the Amazon region in the North, and 10 from northeastern Brazil.

Each sample was divided into two parts; one section was used to isolate O1 phages using MAK 757 (ATCC 51352) as a propagating strain. The other portion was used for non-O1 phages; the non-O1 *V. cholerae* strain was used for propagation starting from O2 to O139. The procedure was repeated on nutrient agar for the appearance of plaques. The phages were purified from a single discrete plaque by the soft agar (0.8%) overlay method with the propagating strain of *V. cholerae* non-O1 (O2 to O139) until homogenous plaques were obtained. Phage lysates were prepared in nutrient broth (Difco Laboratories, Detroit, MI) with the propagating strain *V. cholerae* (both O1 and non-O1).

High-titer phages were obtained by the plate lysis procedure by using the agar overlay method (5) with multiplicity of infection 0.01 at an incubation temperature of 37°C. Concentration, purification, and electron microscopy study of these phages were performed as described by Ghosh et al. (1989) (7), using a FEI Tecnai 12 Bio Twin transmission electron microscope (FEI Europe BV, Eindhoven, Holland, the Netherlands). Measurements were made with Analysis (SIS GmbH, Munster, Germany) software. The homogeneity of each phage was studied by plaque morphology on nutrient agar following 10-fold serial dilution. Each high-titer phage ( $10^8$ – $10^9$  PFU/mL) was then used to determine routine test dilution (RTD) by the soft agar overlay method. In this experiment, the RTD used was the highest dilution that failed to give confluent or complete lysis. A variety of enteropathogens were included for susceptibility against these phages. A single colony from nutrient agar plate was injected into 5 mL of nutrient broth and incubated 2–3 h under stationary conditions at 37°C. A bacterial lawn was made on nutrient agar with this broth culture mixed with 3.5 mL of molten soft agar. The phage at RTD was spotted onto the plate and incubated at 37°C for 18 h. The next morning, the appearance of the zone of lysis was recorded. *V. cholerae* MAK 757 (ATCC 51352) was included as a positive control.

Of the 32 samples examined, two non-O1 phages (O6 and O34) sensitive to *V. cholerae* O6 and O34 were isolated from the same site, the Sarapui River in Rio. The plaques of both the phages were observed as clear and round, with a diameter of 3 to 4 mm. A total of 107 strains of *Vibrio* and *Enterobacteriaceae* were tested against these two phages. All strains were untypeable, except for the O6 and O34 serotypes, which were lysed by the phages specific for

O6 (DR1) and O34 (DR2). During the study period, the recorded temperature was 25°C–38°C, and pH ranged from 8 to 10. No correlation was observed with these two parameters and the isolation of phages.

The morphology of the phages was studied by negative staining electron microscopy. The O34 phages have a hexagonal head with a long tail. The diameter (distance between opposite apices) of the head is  $83.0 \pm 0.3$  nm, while the length and width of the tail are  $111.0 \pm 0.8$  nm and  $17.0 \pm 0.5$  nm, respectively (Figure 1). The O6 phage has a similar form, with a head diameter of  $77.5 \pm 0.3$  nm and a long tail  $100.0 \pm 0.6$  nm in length and  $19.0 \pm 0.4$  nm in width (Figure 2). The other enteropathogens isolated from the sewage samples were enteropathogenic *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. (data not shown).

The use of vibriophages as tools for studying the source or origin of *V. cholerae* has contributed greatly to the understanding of the epidemiology of this disease. The emergence of *V. cholerae* O1 in South America in 1991 provoked major social and economic damage. A total of 60,000 clinical cases were reported from 1990 to 1996 in the coastal city of Rio de Janeiro. In 1997, approximately 2,600 confirmed cases of cholera were reported. In 1998, the number of cholera cases was 376. In 1999, the number of cases increased to 4,142; in 2000, the number decreased to 821 cases. This extreme variation in cholera cases continued during 2001, 2002, and 2003 (until February): the number of cholera cases reported were 665, 174, and 10, respectively. However, since 1993, no cholera cases caused by O1 have been reported. Only cases of non-O1 have been encountered, with O6 and O34 the predominant serotypes. A nationwide survey conducted by the National Reference Center for Cholera under Instituto Oswaldo Cruz is ongoing to

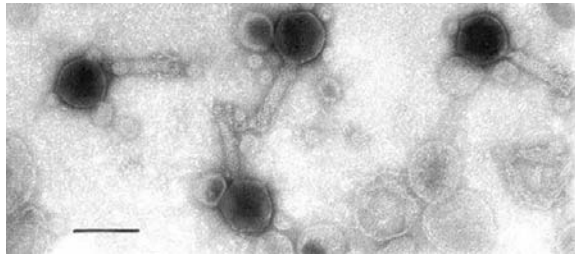


Figure 1. Electron micrograph of O34 vibriophage. Bar represents 100 nm.

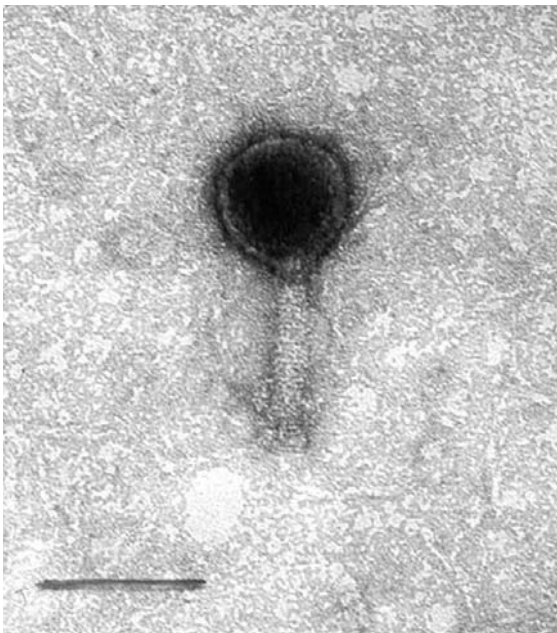


Figure 2. Electron micrograph of O6 vibriophage. Bar represents 100 nm.

isolate more phages in Brazil and neighboring countries.

To date, serotyping is the only identification tool for the characterization of non-O1 strains of *V. cholerae* (8). However, serotyping is only performed at a limited number of laboratories. For this study, all isolates from Brazil were sent to laboratories outside the country for serotyping. This step was expensive and time-consuming and posed risks during transit.

An alternative method is the use of phages for identifying non-O1 strains. This method offers an affordable monitoring system in less-developed countries such as Brazil. Phage O6 and O34 should at least be useful for confirming the diagnosis of *V. cholerae* O6 and O34 infection and for differentiating *V. cholerae* O1 and non-O1 strains.

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## *Salmonella* Agona Harboring Genomic Island 1-A

**To the Editor:** Multidrug-resistant *Salmonella enterica* serovar Typhimurium definitive phage type 104 has emerged during the 1980s and 1990s as a world health problem because of its implications in animal and human disease (1-3). Epidemic serovar Typhimurium definitive phage type 104 isolates are commonly resistant to ampicillin (Ap), chlo-

ramphenicol (Cm)/florfenicol (Ff), streptomycin (Sm)/spectinomycin (Sp), sulfonamides (Su), and tetracyclines (Tc) (1,3). This multidrug-resistance phenotype is conferred by an antibiotic resistance gene cluster included in a 43kb genomic island named *Salmonella* genomic island 1 (4). *Salmonella* genomic island 1 has been recently characterized and located between the *thdF* and *int2* genes of the chromosome. The *int2* gene is part of a retron sequence found only in serovar Typhimurium. Downstream of the retron sequence is the *gidY* gene, which is also found in the chromosome of other *S. enterica* serovars. The antibiotic resistance gene cluster of approximately 13 kb is located at the 3' end of *Salmonella* genomic island 1 (4). All resistance genes are clustered and are bracketed by two integron structures (5,6). The first integron carries the *aadA2* gene, which confers resistance to Sm and Sp. The second integron contains the  $\beta$ -lactamase gene *pse-1*, conferring resistance to Ap. Flanked by these two integron structures are the *floR* gene, which confers cross-resistance to Cm and Ff, and the tetracycline-resistance genes *tetR* and *tet(G)* (5,6). Recently, *Salmonella* genomic island 1 has also been identified in other serovars of *S. enterica* namely Agona (4,7), Paratyphi B (8), and Albany (9), indicating the horizontal transfer potential of *Salmonella* genomic island 1. In these serovars, *Salmonella* genomic island 1 has the same chromosomal location as in serovar Typhimurium definitive phage type 104, except that they lack the retron sequence found downstream of *Salmonella* genomic island 1 (4,8,9). Moreover, six variant *Salmonella* genomic island 1 antibiotic resistance gene clusters (*Salmonella* genomic island 1-A to -F) have recently been reported for serovars Typhimurium DT104, Agona, and Albany to confer different multidrug resistance phenotypes (9,10). These clusters of genes were

probably generated after chromosomal recombinational events or by antibiotic resistance gene cassette replacement in the integron structures. In particular, the *dfrA10* gene coding for trimethoprim (Tm) resistance was found downstream of the *pse-1* integron in a third unusual integron structure involving orf513 in the variant antibiotic resistance gene cluster called *Salmonella* genomic island 1-A (ApCmFfSmSpSuTcTm) (10).

Multidrug-resistant serovar Typhimurium definitive phage type 104 was disseminated globally with several outbreaks in humans and animals. At present, in contrast to the world health problem of multidrug-resistant serovar Typhimurium definitive phage type 104, human cases of infections with other *S. enterica* serovars harboring *Salmonella* genomic island 1 have not yet been reported. *Salmonella* genomic island 1-multidrug-resistant serovars Agona, Paratyphi B, and Albany were isolated from different animal species and countries (7–9). In this study, we analysed the first *Salmonella* genomic island 1 positive serovar Agona strain (02/01177) isolated from a human case in Belgium.

A Belgian patient, who had been infected by a multidrug-resistant serovar Agona strain was travelling to Turkey; subsequent to the multidrug-resistant serovar Agona strain, gastroenteritis developed. While in Turkey the patient sought medical care and was treated unsuccessfully with antimicrobial agents. Upon his return to Belgium, this serovar Agona strain was isolated from his stools, and he recovered after treatment with ciprofloxacin. The serovar Agona strain 02/01177 displayed the multidrug resistance profile ApCmFfSmSpSuTcTm, which suggested the possible occurrence of *Salmonella* genomic island 1-A (10). Moreover, the strain showed the same level of resistance to Ff as *Salmonella* genomic island 1 harboring *S. enterica*

serovars (MIC of 64  $\mu$ g/mL) (7–9).

To assess the presence of *Salmonella* genomic island 1 and its location in the chromosome, polymerase chain reactions (PCRs) were performed using primers corresponding to the left and the right (with or without retron) *Salmonella* genomic island 1 junctions to the chromosome as described previously (4,8–10). PCR results were positive for the left junction between the *thdF* gene of the chromosome and the *int* gene of *Salmonella* genomic island 1 (4). For the right junction, PCR results were positive between open reading frame (ORF) S044 of *Salmonella* genomic island 1 and *gidY* gene of the chromosome. Thus, these data indicate that this serovar Agona human isolate contains *Salmonella* genomic island 1 at the same chromosomal location as in other *Salmonella* genomic island 1 positive serovars but lacks the retron sequence found to date only in serovar Typhimurium strains (4,8,9).

PCR mapping of the typical antibiotic resistance genes and integrons associated with *Salmonella* genomic island 1 was realized as described previously (4,8–10). PCR amplifications on genomic DNA extracted from serovar Agona strain 02/01177 yielded all specific fragments of the sizes expected from DNA of serovar Agona control strain 1169SA97 harboring *Salmonella* genomic island 1-A (data not shown) (10). These PCR mapping results indicated the presence of the typical *Salmonella* genomic island 1 resistance gene cluster with the insertion of the third unusual orf513 integron structure carrying *dfrA10* (8–10). These data are in accordance with the multidrug resistance phenotype of serovar Agona strain 02/01177 and indicate the presence of the variant antibiotic resistance gene cluster *Salmonella* genomic island 1-A (10).

Macrorestriction analysis by pulsed-field gel electrophoresis of DNA from serovar Agona strain 02/01177 cut by *Xba*I or *Bln*I, showed



that this human isolate is indistinguishable by its *Xba*I or *Bln*I macrorestriction patterns from the other multidrug-resistant *Salmonella* genomic island 1-carrying serovar Agona strains isolated from poultry in Belgium (data not shown) (7). Thus, the human serovar Agona isolate appears clonally related to those from poultry.

To our knowledge, this is the first report describing a human infected by a serovar Agona strain harboring *Salmonella* genomic island 1-A. Moreover, it shows the first case where another *S. enterica* serovar harboring *Salmonella* genomic island 1 than the epidemic serovar Typhimurium definitive phage type 104 clone is implicated in human infection. The patient could probably have been infected before his travel to Turkey by a *Salmonella* genomic island 1-A carrying serovar Agona strain in Belgium where this type of strain is frequently isolated from poultry (Doublet et al., pers. comm.). This hypothesis is also supported by macrorestriction analysis, which showed that the strains from poultry and the human case-patient had identical *Xba*I and *Bln*I pulsed-field gel electrophoresis patterns and thus indicate that they are clonally related. Moreover, the patient was not in contact with poultry during his stay in Turkey and, to date, very little is known about the epidemiology of multidrug-resistant serovar Agona strains in this country. Further investigations on the epidemiology of multidrug-resistant serovar Agona strains harboring *Salmonella* genomic island

1 are warranted to avoid such strains becoming a worldwide epidemic, as was the case for multidrug-resistant serovar Typhimurium definitive phage type 104.

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## Conference Summary

### Consequences of Bacterial Resistance to Antimicrobial Agents

In December 2002, a colloquium was organized by the Institut Pasteur and the Institut de Veille Sanitaire, Paris, France, to review what current knowledge exists on the impact of antimicrobial bacterial resistance and address the methodologic obstacles to its assessment.

Simply to state that a patient died of an infection caused by a resistant organism does not prove that the death was due to the resistance. To prove the statement as to cause of death, two approaches—"imputable death" and "attributable death"—are complementary and have been shown to provide comparable results. The estimation of "imputable death" requires analyzing the clinical history of a series of deaths that were caused by resistant strains of infection and to count by clinical judgment those related to the resistance. To estimate the "attributable" fraction of death or illness, the excessive risk for death or illness must be documented for patients who are infected with the resistant strains, in comparison with those who are infected with sensitive strains. The study design must control for confounders by matching the groups at inclusion or by adjustment. Among these confounders the most important are the severity of the underlying illness before onset of infection, which may be associated with both the risk for death and the risk of antibiotic resistance of the bacterium. The time at which death is evaluated is another key issue of the study design. An excessive rate of mortality may be observed during the first months of follow-up and not during a longer follow-up.

Multidrug- (isoniazid and rifampicin) resistant tuberculosis (MDRTB) is associated with a more than threefold increased death if an appropriate anti-TB regimen is not used early in the course of the infection. In western countries where the prevalence of MDRTB is low and second-line drugs are available, MDRTB only requires prompt detection and adequate management to limit the consequence of resistance. However, in developing countries where second-line treatments are not readily available, and where 95% of worldwide tuberculosis cases occur which are responsible for 26% of the potentially avoidable death, one can predict an increasing impact of MDRTB on death in the years to come. Several studies suggest that in acute otitis media caused by *Streptococcus pneumoniae*, the bacteriologic failure rate increases with penicillin G MICs. Although bacteriologic failure does not mean clinical failure, the risk for acute otitis media relapses and complications linked to resistance is poorly documented. Higher penicillin G MICs of *S. pneumoniae* strains observed in mastoiditis than those in otitis and sinusitis does not prove that the severe acute otitis media complications increase with *S. pneumoniae* resistance. Whether invasive infections with resistant *S. pneumoniae* strains are linked to excessive death rates remains controversial. Two studies suggest that death may be greater with higher levels of resistance to penicillin G, and several failures have been reported with macrolide or fluoroquinolone therapy. In countries with high levels of drug resistance and where multidrug resistance is frequent, such as southwestern Europe, the likelihood of treatment failure in meningitis or mastoiditis might be greater. Because widespread use of pneumococcal conjugate vaccine has been shown to reduce the risk for resistant infections, epidemiologic studies to evaluate potential benefits

of conjugate vaccine introduction are needed in countries most affected by resistance.

The quinolone resistance in bacterial diarrhea due to *Campylobacter jejuni* can lead to therapeutic failure associated with an increased duration of symptoms and an increased rate of hospitalization. For non-typhi *Salmonella*, resistance was associated with an increased rate and duration of hospitalization, a twofold increased risk of death during a 2-year period after the infection, and an increased rate of invasive infection (1). Antimicrobial use may cause a transient decrease in a person's resistance to colonization by noncommensal bacteria as well as infection upon exposure to a food-borne pathogen. The additional selective effect of antimicrobial resistance results in a greater than threefold increase in vulnerability to infection by an antimicrobial-resistant pathogen among persons receiving antimicrobial therapy for unrelated reasons. The net result, which has been demonstrated for salmonellae and campylobacters, is an excessive rate of illness caused by the interaction between resistance in these bacteria and unrelated use of antimicrobial agents in humans. This relationship may also explain why outbreaks of resistant food-borne agents which lead to an excess illness among immunocompromised persons or persons at risk, may be more common in hospitals than in communities. Studies of the clinical outcome of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in comparison to methicillin-sensitive *S. aureus* (MSSA) infections have produced conflicting results. A dozen studies compared MRSA and MSSA infections of the same infection site with adjustment for at least one recognized criterion of illness severity and included at least 30 patients. In studies of MRSA bacteremia in which the analyses took into account the presence of shock, the source of the

infection, underlying disease(s), the medical setting in which the infection occurred, the appropriateness of antibiotics prescribed, the age and sex of the patients, no increased death was associated with MRSA, although inappropriate therapy was associated with a poorer outcome. In contrast, for bone infections and mediastinitis, MRSA may increase the risk of death.

The complete results of standard antimicrobial susceptibility tests are not generally available to the prescriber before at least 48–72 hours. The initial regimen prescribed may be not adequate during the first 2 to 3 days of treatment. This may impact death or illness attributable to multi-resistant bacteria. Shortening this interval, rapid diagnosis techniques based on molecular identification of resistance mechanisms could improve outcome. For example, methicillin resistance in *S. aureus* colony is detectable within 6 hours. Studies on clinical specimens showed that resistance-detection techniques, coupled with DNA identification of the bacterium, gave an excellent concordance to discriminate MRSA and MSSA and for MDRTB. Advances in the field of DNA microchips might soon improve the clinical impact of these techniques.

With more clinical failures, more expensive alternative regimens, the cost-effectiveness ratio of the treatment of antimicrobial bacterial resistant infections will inevitably rise. However, very few studies have addressed this issue; it requires precise and documented scenarios based on close collaboration between clinicians, microbiologists, epidemiologists and economists. Proposing prospective scenarios and foreseeing all the public health consequences of antimicrobial bacterial resistance is difficult. Although the impact on life expectancy should remain relatively limited in western nations, this will not be the case in developing countries where alternative regimens are

usually either not available or too costly.

Quantifying the consequences of antimicrobial bacterial resistance is a key element for allocating resources for public health programs. Some evidence exists of such consequences on illness and death, most of which appear to be associated with inappropriate or delayed therapy. Nevertheless, more studies which take into account the specific methodologic difficulties mentioned above are needed to better convince policy makers.

**Jean Claude Desenclos\*  
and Didier Guillemot†, on behalf of  
the conference speakers**

\*Institut National de Veille Sanitaire, Saint Maurice, France; and †Institut Pasteur, Paris, France

**Reference**

1. Helms M, Vastrup P, Gerner-Smidt P, Molbak K. Excess mortality associated with antimicrobial drug-resistant *Salmonella typhimurium*. *Emerg Infect Dis* 2002; 8:490-5

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## Vets, Meds, and Zoonotic Threats

The fourth international conference on emerging zoonoses (September 18–21, Ames, Iowa, USA) brought together 180 scientists and healthcare specialists from 18 countries working to control diseases transmitted from animals to humans. The meeting took place under the auspices of the Center of Food Security and Public Health, USA, and the Institute for International Cooperation in Animal Biologics (a collaborating center of the World Animal Health Organisation [OIE]).

A multidisciplinary and global approach shed new light on both old and new zoonoses. For example, brucellosis topics covered a wide range of material, from economic aspects of control in Mongolia to characterization of *Brucella* isolates from feral swine in coastal South Carolina. Another presentation concerned the increasingly appreciated role of wildlife in the dynamic epidemiology of other zoonotic infections, such as tuberculosis. Scientists also explored the intricate routes prions follow between wildlife and domestic animals; between sheep, cattle, and humans; and between the tongue and brain of infected animals.

Since most agents of bioterrorism potential are zoonotic, a full session was dedicated to bioterrorism and biodefense. It included a global view, a report on national preparedness by Israeli hospitals, and examples of research that may eventually help experts coping with bioterrorism but would also unfortunately be accessible to persons with malicious intent.

Innovative methods for preventing spread of foodborne pathogens were presented, including the use of fluorescence spectroscopy to detect fecal contamination on animal carcasses or the use of vaccination to reduce transmission of zoonotic pathogens and drug-resistant nonpathogens through the food chain to humans. In the field of xenotransplantation, key components of a source-animal production facility were described. The feasibility of breeding pigs free of designated pathogens offers hope for wide use of xenotransplantation in the near future.

Participants also discussed current trends and challenges of protozoan parasitic zoonoses, including cryptosporidiosis, toxoplasmosis, African and Latin American trypanosomiasis, and leishmaniasis. Controversial zoonotic viruses were given an important place in the conference. These included hepatitis E virus, with similar strains causing liver disease in

swine and humans; Borna disease virus, causing neurologic disease in various species of animals as well as, debatably, psychiatric disorders in humans; and the recently discovered severe acute respiratory syndrome-associated coronavirus and its yet-undefined animal reservoir. The recent mapping of the genome of *Mycobacterium avium* subspecies paratuberculosis, the etiologic agent of Johne's disease in cows, brought some hope in solving the long-lasting dispute on its role in the pathogenesis of Crohn's disease in humans.

The value of using a global, multi-disciplinary approach was highlighted in studies on the flow of genes among avian, swine, and other influenza viruses and on the ongoing interconti-

mental spread of arboviruses, exemplified by the evolving epizootic of equine West Nile encephalitis in the United States. Several papers dealt with the epidemiology of Nipah, Ebola, monkeypox, rabies, and Hantaan viruses.

A series of presentations demonstrated how genomic fingerprinting and other sophisticated molecular biology techniques allow exceptionally fast development in understanding the epidemiology and pathogenesis of many zoonotic infections, such as those caused by *Escherichia coli* O157:H7 or by species of *Anaplasma*, *Bartonella*, *Borrelia*, *Campylobacter*, *Coxiella*, *Francisella*, *Pasteurella*, and *Salmonella*.

The "one-track" meeting, by avoiding parallel and superspecialized sessions, gave an opportunity for fruitful and inspiring interactions among experts from multiple disciplines with a shared goal of mitigating human disease from emerging infections. More details on the meeting can be viewed online (available at: <http://www.zoonoses2003.com>).

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#### Correction, Vol. 10, No. 3

On p. 519, in the table entitled "Characteristics of enterotoxigenic *Escherichia coli* (ETEC) outbreaks, United States, 1996–2003," the serotype of the strain associated with outbreak number 16 was O169:H41 not O169:H49.

#### Emerging Infectious Diseases Policy on Corrections

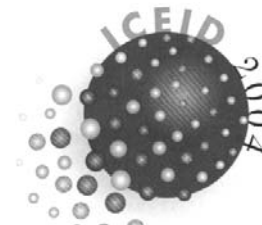
The Emerging Infectious Diseases journal wishes error-free articles. To that end, we

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.

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## Conference Presentations Online and on CD-ROM

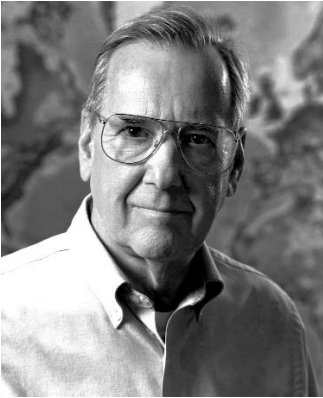


Webcasts—some enhanced by the visual presentations—of the International Conference on Women and Infectious Diseases (February 27 - 28, 2004) and the International Conference on Emerging Infectious Diseases (February 29 - March 3, 2004) are now available.

Online access to the presentations is available to conference attendees. A username and password are required, previously emailed to all registrants. The online sessions are accessible for a limited time only.

Conference presentations are also available on CD-ROM, which includes audio for selected sessions and visual elements for all sessions. A CD-ROM may be purchased for ICWID (US \$99.00 plus shipping) and for ICEID (US \$129.00 plus shipping) from Conference Archives. Call 1-866-316-3289 or 1-814-536-3356 (outside the United States) to purchase. Additional information is available at [www.conferencearchives.com/icwid](http://www.conferencearchives.com/icwid) and [www.conferencearchives.com/iceid](http://www.conferencearchives.com/iceid)

## Robert Ellis Shope



1929–2004

**R**obert Ellis Shope, one of the world's most distinguished arbovirologists and a dear friend of many colleagues around the world, died of complications of idiopathic pulmonary fibrosis in Galveston, Texas, on January 19, 2004, at age 74. Bob is survived by his wife, Virginia; his daughters, Deborah Shope and Bonnie (Shope) Rice; his sons, Peter and Steve; his brothers, Thomas and Richard; his sister, Nancy (Shope) FitzGerrell; and six grandchildren.

It is difficult to describe Bob's many contributions to virology, epidemiology, tropical medicine, infectious disease sciences, vector biology, and international public health because they are so numerous and varied. His lifelong contributions to our understanding of arthropod-borne viruses, hemorrhagic fever viruses, and the diseases these viruses cause are without equal. He discovered and characterized more previously unknown viruses than any other person in history. Working at various times in nearly every country where these viruses and diseases are important, he collaborated with virtually everyone who has worked in these fields in the past 50 years. Until his death, he remained an international leader in framing the global response to emerging and reemerging diseases and our national response to bioterrorism, while at the same time keeping

his own laboratory productive—his research was funded continuously by the National Institutes of Health (NIH) for 26 years.

Arguably, Bob's most important contribution was his co-chairing, along with Joshua Lederberg and Stanley Oaks, of the Institute of Medicine Committee on Emerging Microbial Threats to Health. The proceedings of this committee led to the publication in 1992 of *Emerging Infections: Microbial Threats to Health in the United States* (National Academy Press). This seminal publication, which outlined factors implicated in the emergence of infectious diseases and the programs and resources needed to cope with them, initiated much of the current worldwide interest in infectious diseases. He then spent endless days explaining the concepts underpinning the report in order to gain public and political support. His efforts were marked by great success, as evidenced by the revitalized state of the infectious disease sciences today.

Bob was born in Princeton, New Jersey, the son of Richard Shope, an internationally renowned virologist. He received BA and MD degrees from Cornell University and completed an internship in internal medicine at Grace-New Haven Hospital (Yale University School of Medicine). He then spent 3 years in the U.S. Army Medical Corps, where he was initially assigned to Camp Detrick (now the U.S. Army Medical Research Institute for Infectious Diseases) and later to the U.S. Army Medical Research Unit in Kuala Lumpur. The latter experience, involving studies on the etiology of fevers of unknown origin among British soldiers and the local Malaysian civilian population, had a profound effect on his subsequent research interests and career decisions. After starting a residency in internal medicine at Yale, he left to take a staff position with the Rockefeller Foundation's Inter-

national Virus Program in its laboratory in Belem, Brazil (now the Instituto Evandro Chagas). There he remained for 6 years, eventually serving as director of that institute. This was a time of great excitement and discovery, as many new viruses were being isolated and characterized. In 1965, Bob returned from Brazil to Yale, where most of the senior staff of the Rockefeller Foundation's overseas virus program had relocated and were establishing the Yale Arbovirus Research Unit (YARU). Bob remained at Yale for 30 years, rising to the rank of professor and director of that research unit.

In 1995, Bob moved to the University of Texas Medical Branch in Galveston, where he held several appointments: professor (Department of Pathology, Department of Microbiology and Immunology, Department of Preventive Medicine and Community Health), associate director of the university's Center for Biodefense (and John S. Dunn Distinguished Chair in Biodefense), and member (Sealy Center for Environmental Health & Medicine, Sealy Center for Structural Biology, and World Health Organization [WHO] Collaborating Center for Tropical Diseases).

At various times throughout his long career, Bob served as president and councilor, American Society of Tropical Medicine and Hygiene; chair and member, Advisory Council, James A. Baker Institute for Animal Health, Cornell University; member, WHO Expert Panel on Virus Diseases, and member U.S. Delegation to the U.S.–Japan Cooperative Medical Science Program, International Committee on Taxonomy of Viruses (ICTV), Armed Forces Epidemiology Board, Advisory Board of the Fogarty International Center, National Institutes of Health, Institute of Medicine Committee on Improving Civilian Medical Response to Chemical and Biological Terrorism



Incidents, Advisory Committee (American Museum of Natural History Infectious Disease Exhibition), Advisory Panel (National Research Council Program on Strategies to Protect the Health of Deployed U.S. Forces), and the National Research Council Committee on Climate, Ecosystems, Infectious Diseases, and Human Health.

Over the years, Bob earned many honors, including the Bailey K. Ashford Award from the American Society of Tropical Medicine and Hygiene, the Richard M. Taylor Award from the American Committee of Arthropod-Borne Viruses, and the Walter Reed Medal from the American Society of Tropical Medicine and Hygiene.

In November 2003, the University of Texas Medical Branch celebrated the completion of a biosafety level 4 laboratory, the first in the United States at an academic institution. In Bob's honor, the laboratory was named "The Robert E. Shope, M.D. Laboratory." Also in his honor, the university established the Robert E. Shope, M.D. Memorial Fellowship in emerging viral diseases research.

### Professional Odyssey

Bob's first assignment with the Rockefeller Foundation in New York in 1954 was to study the etiology of "epidemic polyarthritides," a disease characterized by fever, arthralgia, and rash that occurred mainly during the summer in coastal regions of Australia. In a classic retrospective serologic investigation, he and S.G. Anderson demonstrated that the disease was caused by an alphavirus. Ten years later, Australian scientists, led by Ralph Doherty and Ian Marshall, isolated Ross River virus and confirmed its association with epidemic polyarthritides.

In the early 1960s, during Bob's tenure at what is now the Instituto Evandro Chagas, he isolated and char-

acterized more than 50 tropical arboviruses, most of which were new to science. Several, such as group C and Guama viruses, caused human disease. Not only did he play a role in isolating and characterizing these agents and their diseases, but his team was instrumental in studying and understanding the role of their forest reservoirs. He refined the capture-mark-release-recapture technology for rodents, marsupials, and birds, and applied these methods to the Amazon fauna. His team also correlated vertebrate reservoir ecology with that of vector mosquitoes. He identified Oropouche virus when the first epidemic struck the city of Belem. This disease has since emerged as a major scourge, not only in the Brazilian Amazon, but also in cities in Panama and Peru.

During his early tenure at the Yale Arbovirus Research Unit, Bob was involved in a collaborative project with the Smithsonian Institution and discovered several new arboviruses transported by birds migrating through the Nile Delta. Then, in 1969, Lassa fever and yellow fever emerged in Nigeria and occupied his research for several years.

Bob was first to show that rabies virus was related to other viruses (the "rabies-related viruses"); in 1970, with Fred Murphy and others, he characterized Mokola, Lagos bat, and Duvenhage viruses, the founding members (along with rabies virus) of the genus *Lyssavirus*, family *Rhabdoviridae*. In 1971, Bob, Fred Murphy, and Ernie Borden characterized many bluetongue-like viruses and established the genus *Orbivirus*, family *Reoviridae*. In 1971, Bob coauthored an obscure paper in the Indian Journal of Medical Research describing Thottapalayam virus, a presumed arbovirus isolated from a shrew in India. Thottapalayam virus was subsequently shown to be a hantavirus, the first hantavirus to be isolated (Hantaan virus, the type virus of

the genus *Hantavirus*, family *Bunyaviridae*, was not isolated until 1976).

In 1977, Bob identified a virus isolated from a mixed pool of midges (culicoids) collected near Darwin, Australia, and sent to him by Toby St. George. Bob identified it as bluetongue 20 virus, the first bluetongue virus recognized in Australia. This discovery caused economic turmoil in the Australian livestock industry, but it was seminal in initiating an intensive research program in that country. That same year, Bob and Jim Meegan identified Rift Valley fever virus as the cause of a "virgin soil" epidemic in Egypt that affected 200,000 people, with more than 600 human deaths, and hundreds of thousands of sheep and cattle deaths. Also noteworthy is that in 1977, Bob co-authored the first description of Lyme disease in the United States. This work was done with Allan Steere, then a young rheumatologist at Yale. Lyme disease was subsequently recognized as the most important tick-borne disease in North America.

Throughout the most recent 20 years, Bob and his colleagues, especially Bob Tesh, continued to discover new, important viruses. These included Sabiá, the cause of Brazilian hemorrhagic fever, and Guanarito, the cause of Venezuelan hemorrhagic fever. Bob also worked to develop an attenuated-live virus vaccine for dengue and chaired the WHO advisory committee on dengue vaccine development.

After moving to the University of Texas Medical Branch in 1995, Bob embarked on a new phase of his career; he and his colleagues initiated a new program on bioterrorism countermeasures, centered on novel antiviral drugs against alphavirus, flavivirus, and arenavirus diseases. At the same time, along with Bob Tesh, Bob brought the World Reference Center for Emerging Viruses and Arboviruses to the university—this is

one of the most valuable virus collections in the world, comprising >4,000 arthropod-borne virus strains and >1,000 other virus strains.

### Scientific Legacy

In addition to the many scientific contributions already mentioned, Bob wrote and edited more than 170 refereed papers and more than 90 books, chapters, and monographs. Most notable of his editorial activities was his contribution to several editions of the definitive reference book in virology, *Fields Virology* (Bernard N. Fields, editor; David Knipe, Robert Chanock, Joseph Melnick, Bernard Roizman, Robert Shope, Martin Hirsch, Thomas Monath, Peter Howley, and Stephen Straus, co-editors; Lippincott Williams & Wilkins).

Bob's oral contributions are legion. Over the years, he presented many hundreds of seminars, lectures, and workshops throughout the world, but he never kept track of them. One can only guess at the numbers, but it is easy to recall their overall impact in many disciplines of medical science.

### Legacy as a National and International Advisor and Consultant

Bob served the national and international interests of science in many ways—he was an outstanding spokesman, always willing to take extra time and effort to “educate” politicians and science leaders. He did this at an ever-higher level of impact: in 1997 Bob was invited to the White House along with six other scientists, three of them Nobel laureates, to brief President Bill Clinton, Vice President Al Gore, and others on the perils of global warming and its importance in the spread of infectious agents, such as those causing dengue and dengue hemorrhagic fever, malaria, and many other arthropod-borne diseases.

Bob served as consultant and advisor to many national and international organizations. Throughout his career

he was a frequent advisor to the NIH, Centers for Disease Control and Prevention, Department of Defense, U.S. Department of Agriculture, U.S. Agency for International Development, the Institute of Medicine, and the American Type Culture Collection. He was also a frequent consultant to WHO and the Pan American Health Organization, as well as to foreign governments.

### Legacy as a Teacher and Mentor

Bob was an outstanding, devoted, and beloved teacher. He cared deeply about the next generation of professionals entering the various fields of the clinical infectious disease sciences, virology, vector biology, epidemiology, and public health. His honesty, humility, enthusiasm, and caring manner endeared him to his students. He treated everyone with respect and was never too busy to meet with a student or to offer guidance. But there was more; there was a kind of magic in his “touch” as teacher and mentor. For example, Bob participated for many years in the Cornell Summer Leadership Program, run lovingly by Doug McGregor. At the end of a day spent in workshops, the students would gather in a lounge for a beer and informal discussion with the faculty. Within minutes, other guest faculty members were forgotten as the students zeroed in on Bob—they instinctively knew that he was the utmost rolemodel, an inspiration in deciding career directions.

### Legacy as a Colleague and Friend

Over the years, Bob worked with many prominent arbovirologists and vector biologists, including, in his tenure at Yale, Tommy Aitken, John Anderson, Sonia Buckley, Jordi Casals, Delphine Clarke, Wilbur Downs, Max Theiler, Loring Whitman, Jack Woodall, Barry Beaty, Rebecca Rico-Hesse, Dennis

Knudson, Barry Miller, Thomas Schwann, Thomas Scott, Mark Wilson, and others. During his tenure at the University of Texas Medical Branch, he worked closely with Dave Walker, Bob Tesh, C.J. Peters, Stan Lemon, Scott Weaver, Alan Barrett, Judith Aronson, Chuck Fulhorst, Norb Herzog, Steve Higgs, Peter Mason, Doug Watts, Larry Stanberry, and others. But, such lists are folly—Bob's true legacy as a colleague and friend would require a list of hundreds of names, from across the years and around the world. It would be wonderful to append such a list here, but it would be suspect in its likely omissions.

The essence of Bob's legacy as a colleague and friend is the most difficult matter to capture here. After his death, condolences to his colleagues and family contained oft-repeated themes:

“He was the nicest, humblest, most self-effacing, and obliging person I have ever known...”

“He was unfailingly kind and generous...”

“He was always collegial with collaborators and endlessly patient with students; it is not surprising that he earned both the respect and love of both...”

“He consistently exemplified and fostered an attitude of service...”

“He always made time for you, whether you were a student or the minister of health...”

“He was always reassuring, upbeat and helpful...”

“He was an exceptionally generous person, and that tended to rub off on others...”

“His enthusiasm and love for his chosen field were as infectious as the viruses he studied...”

“Those of us who knew him and worked with him were very fortunate...”

“He was a catalyst of honesty, professionalism, and mutual respect....”

“We all benefited from his example...”

“He was the best listener in the world...”

“He always acted in the best interests of others, truly practicing the golden rule of treating others as he would wish to be treated...”

“We were blessed to have had him as a colleague...”

Running through these messages is the understanding that the field of arbovirology and related sciences has been incredibly satisfying to the involved community because of the warmth of personal relationships and the high ethical standard shared by one and all. In these messages, it is clear that everyone knew that Bob was the “keeper of the flame,” the exemplar, the strong positive influence on everyone in this community. This was not a passive matter—in the most subtle way Bob vaccinated others with his high ethical standard—magic, again!

The messages received in Galveston upon Bob’s death capture another theme, one based in each person’s memories of his or her earliest days in the world of medical research and public service. It seems that many, many people have a clear mem-

ory of their first experience with Bob. Two messages, anonymously paraphrased here, are exemplary. First, “...In the 1970s I went to YARU with a few viruses that had been brought back from Kenya and couldn’t be identified. I was new to the field and had little clue how to go about determining what these viruses were. But I had landed in the World Reference Center! Bob took me under his wing. He showed me the reference collection, inventoried on a huge Rolodex. We talked about the possibilities based on the source of the unknown viruses. Bob stayed with me until 10 p.m. developing a testing plan, and then drove me to my hotel. My head was spinning. YARU was a busy place and there were many other much more important visitors, but Bob worked closely with me over the week until we had identified all of the viruses. The result was one of my first publications in arbovirology...”

Second, “...I well remember as a young nobody from the outback of Africa arriving to consult with Bob at YARU in 1975. I wanted some reference arbovirus reagents to take home with me, and having just met me, Bob worked with me late into the evening, freeze-drying viruses and antigens so that I could leave with them on the following day...”

Does all this capture Bob Shope, the man, the friend that we have lost? If not, then words cannot serve what memories can. To say that Bob will be missed is the greatest of understatement—he will never be forgotten. He leaves to us a legacy of enthusiastic commitment to his science and an equal commitment to the application of that science for the benefit of the people of the world. As well, he leaves to us, by the example of his life, a definition of our sense of community and the keys to its nurturing.

Respectfully submitted on behalf of the world’s community of virologists, especially the community of arbovirologists,

**Frederick A. Murphy,\*  
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**Paul Gauguin (1848–1903). I Raro te Oviri (Under the Pandanus) (1891)**

Oil on canvas (73.03 cm x 91.44 cm).

Dallas Museum of Art, Foundation for the Arts Collection, gift of the Adele R. Levy Fund, Inc.

“**B**etween me and the sky there was nothing except the high frail roof of the pandanus leaves, where the lizards have their nests,” wrote Paul Gauguin in the autobiographical account of his first visit to Tahiti (1). Under the Pandanus (on this month’s cover of *Emerging Infectious Diseases*) was painted shortly after Gauguin arrived on the islands in search of his famed reprieve from Western civilization.

Like many of his contemporaries, Gauguin became disillusioned with industrialized society whose intense focus on material gain seemed to strip life of its spiritual essence. Crushed under the yoke of familial responsibility, bewildered by the prosaic rules of art dealing, and stifled by societal constraints, Gauguin imagined a life uncluttered by the tedium of survival. He longed for a different world, one with just enough depth to sustain his most basic needs. Flat and two dimensional, this world would be filled with vibrant color and would celebrate the human spirit long lost under oppressive layers of cultural complexity and control.

While many rebel against civilization and espouse notions of an unspoiled haven, Gauguin set out to embody them in his life and work. A prosperous stockbroker and avid art collector, whose inventory included works by Daumier, Monet, Renoir, Manet, Cézanne, and Pissarro, he abandoned the Paris business scene and his brood of five children to devote his life to art at age 35 (2). Giving up comfort, commercial success, and artistic acclaim, he embraced isolation to know primitive idyll and find the core truth missing from his life.

He sought solace at first in Brittany among the peasants of the French countryside and then in the far away islands of the South Pacific, whose promise of paradise on earth

lured many others, among them, Herman Melville, Mark Twain, and Robert Louis Stevenson. Under the blazing sun of the Polynesian islands, where as he put it in his copious writings, “...the material necessities of life can be had without money” (3), Gauguin articulated his artistic sentiment into original work that influenced generations to come. Synthesizing elements of his admired contemporaries, Cézanne, van Gogh, and others, and inspired by Japanese prints, folk art, and medieval stained glass, he created exuberant tableaux charged with sensuality and primal tension.

Gauguin’s life as adoptive “savage” was one of unrelenting hardship, for the primitive idyll existed only in his inflamed imagination. From the moment he arrived on the islands, he was plagued by two of the many motivators of civilization, poverty and disease. Unable to afford even painting supplies and weakened by malnutrition and syphilis, he moved from Martinique to Tahiti and finally the Marquesas Islands, where he died at age 53.

Gauguin’s art expressed his vision of the world. The edge of the canvas did not frame the images but rather opened them to wider exploration. Unspoiled nature was bountiful and generous, warm, forgiving, and open. Like his paintings, it had no boundaries, and its essence existed only in the imagination. Even as his body failed and his resources expired, its lure did not fray, nor did his zeal for it diminish.

Unlike civilized society, whose joyless monotone had alienated him, the primitive idyll had not been meddled with or manipulated. Unregulated and unrestrained, it followed nature’s rhythm. Painted in startling, unnatural colors that punctuated the spiritual as well as the physical, it had a languid but steady beat. In the moist heat, labori-



ously outlined flat figures of humans and animals shared a communal living, even if it was not, as Gauguin wished it, altogether loving and harmless.

The prickly pandanus (screw pine), whose symbolic abundance pervades the painting on this issue's cover, is native to many Pacific archipelagoes, providing roof, sustenance, adornment, and medicine to generations of islanders (4). Filtering the sea breezes and moderating the tropical heat, the pandanus shelters the underbrush, which contains the complex ecosystem at the heart of the tropics' languid beat.

Undergrowth vegetation in tropical and subtropical areas is home to countless creatures (mammals, reptiles, birds) that sustain sandflies, ticks, fleas, and mosquitoes, whose complex natural cycles flourish in the heat and humidity so central to Gauguin's Eden. Unnoticed and

unpainted, these vectors nurture the dark underpinning of untamed nature, including arthropod-borne disease: in this issue of *Emerging Infectious Diseases* alone, sleeping sickness in Uganda; dengue in Cuba, French Guiana, Bangladesh, and Myanmar; cutaneous leishmaniasis in Colombia; malaria in Western Kenya; West Nile virus in Guadeloupe; murine virus in the Canary Islands.

**Polyxeni Potter**

1. Gauguin, Paul. *Noa, Noa: the Tahiti journal of Paul Gauguin*. California: Chronicle Books; 1994.
2. Quantum Book. *The great masters*. London: Quantum Publishing Ltd; 2003.
3. Janson HW, Janson AF. *History of art*. New York: Harry N. Abrams, Inc.; 2001.
4. *Pandanus tectorius* [cited 2004 Feb]. Available from: <http://www.comfsm/~dleeling/angio/pandanus.html>

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## Upcoming Infectious Disease Activities

### **April 13–15, 2004**

Biodefense Vaccines, Therapeutics  
and Diagnostics: Policy, Funding,  
Development, Testing, Production,  
and Distribution

Hamilton Crowne Plaza  
Washington, DC

Contact: (818) 888-4444

Web site:

[http://www.infocastinc.com/  
bioachome.asp](http://www.infocastinc.com/bioachome.asp)

### **April 13–16, 2004**

International Conference on the  
Control of Infectious Animal Diseases  
by Vaccination

Office International des Epizooties  
Buenos Aires, Argentina

Contact: 33 (0)1 44 15 18 88

email: [oi@oie.int](mailto:oi@oie.int)

Web site: <http://www.oie.int>

### **April 18–20, 2004**

Society for Healthcare Epidemiology  
of America (SHEA) Annual Meeting  
Philadelphia, PA

Contact: SHEA Meetings Dept. (856)  
423-7222 ext. 350

Web site: <http://www.shea-online.org>

### **May 1–4, 2004**

14th European Congress of Clinical  
Microbiology and Infectious Diseases  
(ECCMID)

Prague, Czech Republic

Contact: Administrative Secretariat  
+44 61 686 77 11

email: [info@akm.ch](mailto:info@akm.ch)

Web site:

<http://www.akm.ch/eccmid2004/>

### **May 11–14, 2004**

38th National Immunization  
Conference

Nashville, TN

Contact: Suzanne Johnson-DeLeon  
(404) 639-8225

email: [NIPNIC@cdc.gov](mailto:NIPNIC@cdc.gov)

# EMERGING INFECTIOUS DISEASES

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## Upcoming Issue

Look in the May issue for the following topics:

Hospital Preparedness and SARS

SARS in Hospital Emergency Room

Characteristics and Outcomes of SARS Cases

Genetic Variation of SARS Coronavirus

Ring Vaccination and Smallpox Control

Virulence Factors for Hemolytic Uremic Syndrome

Transmission of St. Louis Encephalitis, Florida

Acute Tick-borne Rickettsiosis, in Russian Far East

Serologic Responses to *Pneumocystis jiroveci*

Genetic Analysis of VISA Isolates

Bovine Spongiform Encephalopathy in Greater Kudu

Endemic Venezuelan Equine Encephalitis

Syndromic Surveillance in Public Health

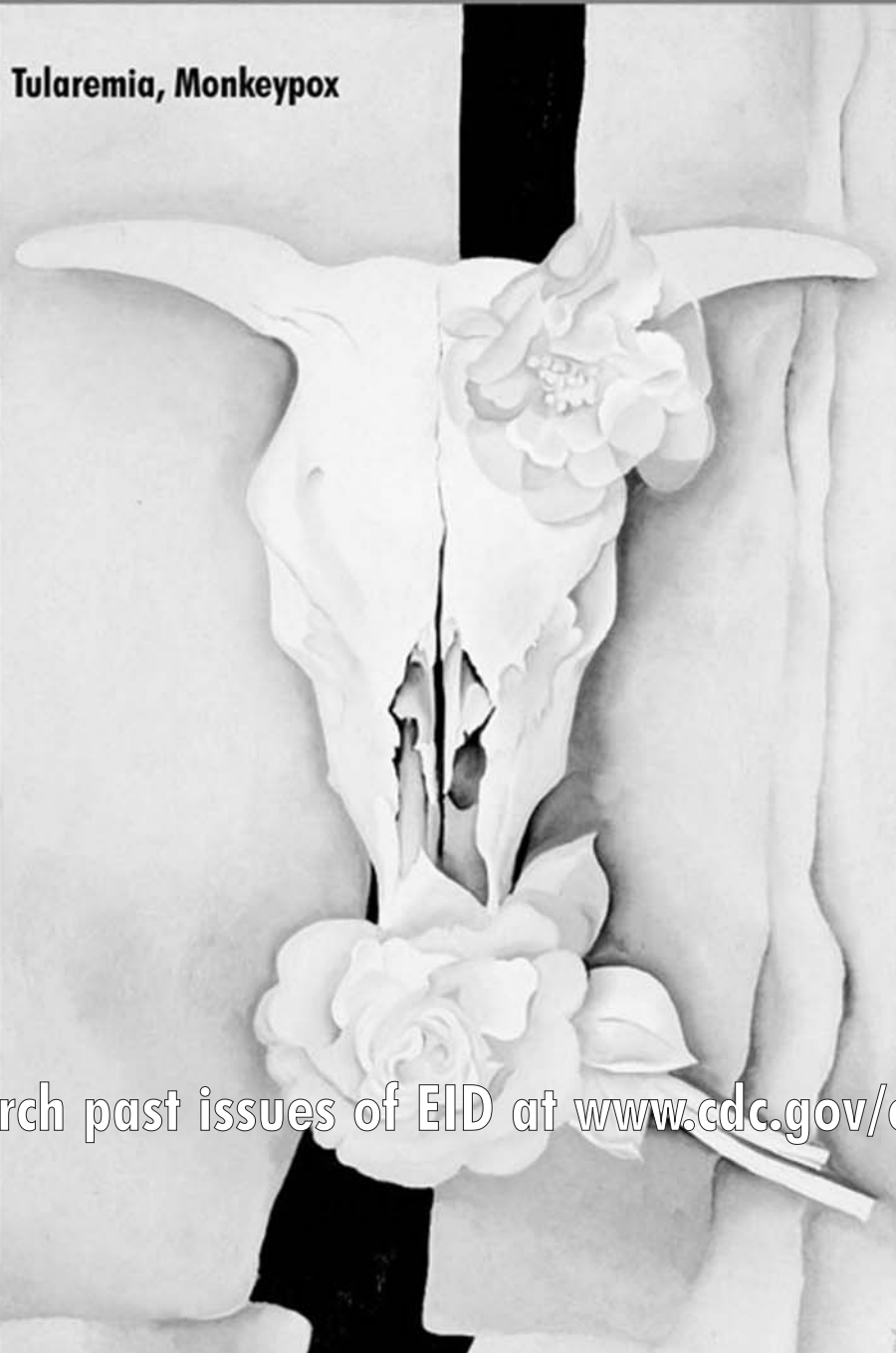
**Complete list of articles in the May issue at  
<http://www.cdc.gov/ncidod/eid/upcoming.htm>**

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## Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or [eeditor@cdc.gov](mailto:eeditor@cdc.gov) (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at <http://www.cdc.gov/eid>.

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## Instructions to Authors

**Manuscript Preparation.** For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

**Title Page.** Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and e-mail address). Include separate word counts for abstract and text.

**Keywords.** Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

**Text.** Double-space everything, including the title page, abstract, references, tables, and figure legends. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

**Biographical Sketch.** Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

**References.** Follow Uniform Requirements ([www.icmje.org/index.html](http://www.icmje.org/index.html)). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

**Tables and Figures.** Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide ([http://www.cdc.gov/ncidod/EID/style\\_guide.htm](http://www.cdc.gov/ncidod/EID/style_guide.htm)).

**Manuscript Submission.** Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website ([www.cdc.gov/eid](http://www.cdc.gov/eid)).

## Manuscript Types

**Perspectives.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Synopses.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch of first author—both authors if only two. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

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

**Policy and Historical Reviews.** Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

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

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

**Another Dimension.** Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

**Letters.** This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**Book Reviews.** Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

**Announcements.** We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

**Conference Summaries.** (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.