EMERGING INFECTIOUS DISEASES

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Risks and Benefits of Preexposure and Postexposure Smallpox Vaccination[1]

Martin I. Meltzer*

This article presents a model and decision criteria for evaluating a person's risk of pre- or postexposure smallpox vaccination in light of serious vaccine-related adverse events (death, postvaccine encephalitis and progressive vaccinia). Even at a 1-in-10 risk of 1,000 initial smallpox cases, a person in a population of 280 million has a greater risk for serious vaccine-related adverse events than a risk for smallpox. For a healthcare worker to accept preexposure vaccination, the risk for contact with an infectious smallpox case-patient must be >1 in 100, and the probability of 1,000 initial cases must be >1 in 1,000. A member of an investigation team would accept preexposure vaccination if his or her anticipated risk of contact is 1 in 2.5 and the risk of attack is assumed to be >1 in 16,000. The only circumstances in which postexposure vaccination would not be accepted are the following: if vaccine efficacy were <1%, the risk of transmission were <1%, and (simultaneously) the risk for serious vaccine-related adverse events were >1 in 5,000.

Smallpox has been identified as a weapon that may be used by a bioterrorist (2,3). Terrorist groups and even nations may have acquired stocks of smallpox produced in the former Soviet Union (4). As a response to this threat, the U.S. federal government has begun to produce and stockpile approximately 300 million doses of smallpox vaccine (2). Properly administered as a preexposure prophylactic, the vaccine is approximately 95%-98% effective. However, smallpox vaccine contains a live virus (vaccinia), and a risk for serious, vaccine-related adverse events exists (5,6). How the stockpile of smallpox vaccine should be used is much debated. Some mathematical models have suggested that, in balancing the risks of a smallpox attack against the risk for vaccine-related adverse events, only healthcare workers need be vaccinated in a preattack situation (7). This phase is essentially the first in the current U.S. federal government's smallpox response plan (8,9).

Others have called for a large-scale, voluntary preexposure vaccination campaign open to the entire U.S. population (10,11). Some concur with such a position in part because they are skeptical that a postattack vaccination-based response will be adequate (12). A telephone survey of the U.S. population, conducted during October to December 2002, found that 61% of the respondents would accept smallpox vaccination if ". . . . offered as a precaution against terrorist attacks" (13). However, despite this trepidation about smallpox, the U.S. federal government's program to vaccinate up to 500,000 healthcare workers and first responders has found that concerns about vaccine-associated risks has caused many to question the need for preexposure vaccination (14–17). Part of this hesitancy includes questions regarding compensation for vaccine-related adverse events (17–19).

This article presents a risk-benefit model of pre- and postexposure smallpox vaccination, which will help public health officials better understand the public's risk-benefit appraisal. Other papers have examined pre- and postsmallpox attack responses from a societal perspective (3,7). The model presented quantifies the perspective of an individual person. The model can be applied to other situations involving pre- and postexposure prophylaxis for infectious diseases (e.g., other vaccines).

Methods

I constructed a risk-benefit model (using a standard computer-based spreadsheet; see online Appendix at URL: http://www.cdc.gov/ncidod/EID/vol9no11/03-0369_spreadsht.xls), which balances the risks for smallpox disease against vaccine-related adverse events (vaccine-related "disease"). The general model is formulated as follows:

Net risk of disease = (risk from smallpox without preexposure vaccination) – (risk of smallpox due to vaccine

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[1]This article presents further methodologic details and results of a study presented at a workshop entitled "Scientific and Policy Considerations in Developing Smallpox Vaccination Options," Instute of Medicine, Washington, DC, 2002 (1).

failure + risk for vaccine-related adverse events from preexposure vaccination) and the precise formula is the following:

Net risk for disease =

$$(P_R \cdot P_E \cdot P_T) - [(P_R \cdot P_E \cdot P_T)(1-P_{VEpre}) + P_{SideEffect} \cdot P_{Valuation}]$$

The symbols and the value for each variable are defined in the Table.

Definitions

The term "disease" refers to case-patients with clinical symptoms caused by either smallpox or serious vaccine-related adverse events. The phrase "serious vaccine-related adverse events" includes death, postvaccinial encephalitis, and progressive vaccinia. Each serious side-effect requires medical care, such as vaccinia immunoglobulin, hospitalization, or a number of visits to a physician's office. In 1968, the rate of postvaccinial encephalitis and progressive vaccinia among first time vaccinees ranged from approximately 0.3 to 1.2 in 100,000 for those aged 1–19 years, 0.7 to 4 in 100,000 for those <1 year of age, and 0 to 1.4 in 100,000 for those >20 years of age (20,21). As most preevent vaccinees are likely to adults, I used a rate of 1/100,000 vaccinees ($P_{SideEffect}$, Table). Vaccine-related adverse events such as eczema vaccinatum, soreness or redness at site of vaccination, headache, and mild and temporary nausea are not considered to be serious vaccine-related adverse events in the model. A risk for eczema vaccinatum occurs in about 1 in 100,000 primary vaccinations (20), which can result in serious consequences requiring intensive medical care, and even (rarely) death (6). I thus underestimate the risk for vaccine-related adverse events, biasing the model toward acceptance of vaccination.

Decision Criteria

If net risk for disease is >0, then the risk for disease from smallpox is greater than the risk for serious vaccine-

Table. Model input variables and values used

Variable	Symbol	Values: Base cases	Values: Sensitivity analyses
Probability of attack	P_R	1:10 –1:100,000	
No. of cases before detection of attack	X_{CASE}	1,000	100,000
General population "at risk"[a]	X_{POP}	9 million or 280 million	
No. of susceptible HCW[b]	X_{HCW}	100,000 or 1,000,000	
Probability of exposure to smallpox, for an	P_E		
Individual member of general populace[c]		1:9,000 or 1:280,000	1:1[j]
Individual HCW contacting infectious person[d]		1:100 or 1;100,000	1:1[j]
Individual member of investigation team[e]		1:2.5 or 1:5	1:1[j]
Probability of transmission of smallpox, for an	P_T		
Individual member of general populace[f]		1.0	0.01–0.70[j]
Individual HCW contacting infectious person[g]		0.70	0.01–0.70[j]
Individual member of investigation team[h]		0.40	0.01–0.70[j]
Probability of vaccine effectiveness, preexposure	P_{VEpre}	0.98[l]	
Probability of serious vaccine-related adverse events[i]	$P_{SideEffect}$	1:100,000	1:500–1:1,000,000[j]
Probability of vaccine effectiveness, postexposure	P_{VEpost}		0.01 - 0.60[j]
Relative individual valuation; case of smallpox Case(s) of serious vaccine related adverse events[k]	$P_{Valuation}$	1:1	1:35

[a]Two populations "at risk" are modeled: a population of 9 million, representing a metropolitan area assumed to be the sole target of a smallpox attack and the entire U.S. population of approximately 280 million. Exactly how a given metropolitan area would be defined as the single target at risk is a matter of speculation.

[b]HCW, healthcare worker.

[c]Risk for exposure for member of the general populace is defined as the risk of contracting, and subsequently developing, a clinical case of smallpox before detection of the event (for individual person in general populace, $P_E = X_{CASE}/X_{POP}$). See text for further details.

[d]Risk of a HCW's becoming exposed is a function of the number of cases divided by number of susceptible HCWs (for HCW, $P_E = X_{CASE}/X_{HCW}$).

[e]Probability of a member of an investigation team being exposed to smallpox includes the probability of being sent to a site where smallpox may be present, such as in a container. There are no data that can be used to accurately define such a risk, and the data used here are assumed.

[f]Probability of transmission of smallpox = 1 indicates that the model only considers those members from the general populace in whom a clinical case of smallpox develops. See text for further details.

[g]Probability of transmission represents when HCWs are not using any effective barrier-type protection (e.g., gloves, gowns, masks). The rate of transmission used, 0.70, is equivalent to the upper estimates of the rates of transmission to unvaccinated household members living with a smallpox patient (Appendix 1 in ref. 2).

[h]Probability of transmission for investigation teams represents a risk after barrier-type protection is used. There are no data representing the actual reduction in risk, and the value of 0.40 is assumed.

[i]Serious vaccine-related adverse events are defined as those adverse events which require "notable" amounts of medical care, such as vaccinia immunoglobulin, hospitalization, or a number of visits to a physician's office. The rate of 1:100,000 is derived from the number of "serious" adverse events (e.g., death, postvaccine encephalitis, progressive vaccinia) measured in 1968 among first-time adult smallpox vaccinees (19,20)

[j]These values are used to examine the risk-benefit of an individual person's accepting smallpox vaccination, including those being revaccinated, for preexposure and postexposure scenarios. See text for further details.

[k]In the base case, it was assumed that a person would value 1 case of smallpox equal to 1 case of serious vaccine-related adverse events. However, a person may be more worried about contracting a clinical case of smallpox than experiencing vaccine-related adverse events. Thus, in the sensitivity analyses, the valuation was altered to reflect a higher valuation of a case of smallpox relative to a case of serious vaccine-related adverse events (see text for further details).

[l] Fenner et al. (22) reviewed five separate studies and reported vaccine efficacy to range from approximately 91% to 97%.

related adverse events, and a person would chose preexposure vaccination. If the net risk for disease is <0, then the risk for serious vaccine-related adverse events is greater than the risk for smallpox, and an individual person would chose no preexposure vaccination.

Scenarios

I use the model to evaluate the net risk for disease faced by a person who is a member of one of the following three groups: 1) The general population. The model compares the risk of being a smallpox patient before an attack is detected to the risk for serious vaccine-related adverse events from preexposure vaccination. The risk of being an actual smallpox patient is modeled by setting the risk for transmission at 1 (Table). Two populations "at risk" are modeled: a population of 9 million, representing a metropolitan area assumed to be the sole target, and the entire U.S. population of approximately 280 million. 2) The healthcare community. For a healthcare worker (HCW) who faces potential exposure to smallpox as a result of caring for a person with smallpox, the risk of contracting smallpox from the patient is compared with the risk for serious vaccine-related adverse events attributable to preexposure vaccination. 3) A smallpox investigation team. For a person who is trained to be deployed to investigate potential patients or attacks (i.e., deliberately seek out potential smallpox patients and material that may be contaminated by smallpox), the risk for contracting smallpox from the patient or other source of smallpox (e.g., aerosol, container) is compared with the risk for serious vaccine-related adverse events from preexposure vaccination. Investigation team members will take precautions to reduce risk for transmission (e.g., wear gloves, face masks, and gowns), reducing risk for transmission to an assumed 0.4 (no data exist regarding the actual reduction in risk attributable to using such barrier precautions).

For all scenarios, after an attack is detected, I assume that appropriate responses will be taken, including effective isolation of patients (2) and vaccination of susceptible contacts. Thus, the results only apply up to the point of discovery of the bioterrorist event.

Sensitivity Analyses

In the model, I assume that persons considering preexposure vaccination value equally the risk for disease from either smallpox or from serious vaccine-related adverse events. In reality, a person may be more worried about contacting a clinical case of smallpox than of experiencing serious vaccine-related adverse events. The risk of dying from smallpox vaccine is approximately 1:1,000,000 vaccinees (20,21), while the death rate due to smallpox may be as high as 30% of all unvaccinated clinical cases (23,24). Using the relative risk of death, I set a comparative value of 1 case of smallpox = 35 cases of serious vaccine-related adverse events ($P_{Valuation}$ = 1/35 = 0.02857).[2] Other sensitivity analyses include increasing the number of cases of smallpox before detection of the attack from 1,000 to 100,000 (Table), and setting the risk for serious vaccine-related adverse events to either 1 in 10,000 or 1 in 1,000,000. The former represents the risk of experiencing probable vaccine-related myocarditis or pericarditis, as measured during the current smallpox vaccination program among civilians (25). The latter is the risk, measured in the 1960s, of serious vaccine-related adverse events (e.g., postvaccinial encephalitis and progressive vaccinia) among revaccinees (20,21).

Risk-Benefit Analysis of Postexposure Vaccination

The model can used to evaluate a person's perspective of the risks and benefits of receiving a smallpox postexposure vaccination. I considered a person who has been exposed to somebody who may or may not have smallpox. To model such uncertainty, I set P_R = 1, and let P_E range from 1 in 10 to 1 in 100,000. I then assumed either a postexposure vaccine efficacy of 10% (P_{VEpost}, Sensitivity analyses, Table) and a risk for transmission of 70% (P_T, Sensitivity analyses, Table), or a postexposure vaccine efficacy of 60% and a risk for transmission of 35%. Additional sensitivity analyses can further vary the values for transmission and efficacy of postexposure vaccination.

I also considered the case in which a person has been exposed to a definite smallpox case (i.e., P_R = 1, P_E = 1). I examined the risk-benefit of postexposure vaccination using a range of risks for vaccine-related adverse events, from 1 in 500 vaccinees to 1 in 100,000 vaccinees. This range encompasses the risks for serious vaccine-related adverse events faced by those without any contraindications for receiving the vaccine, as well as those who would have contraindications for receiving preexposure vaccination (e.g., pregnant women, those with auto-immune diseases, HIV-positive persons). I modeled a "worst case" approach and assumed that postexposure vaccine efficacy would only be 1% (representing, for example, a delay of several days between exposure and being offered the vaccine). The risk for transmission was set at either 1% or 30%, representing possible scenarios, for example, the person considering postexposure vaccination was appropri-

[2]Assume that only a single metropolitan population of 9 million is at risk from an initial attack, and the initial attack results in 1,000 cases before discovery. For a person in that population, the risk for death from smallpox is approximately 33 times greater than the risk for death from the smallpox vaccine [smallpox risk for death/vaccine-related risk for death = (1,000 cases/ 9 million x 0.3)/0.000001]. For a person in a population of 280 million, the risk of dying from smallpox in the initial 1,000 cases is approximately equal to the risk for death from the vaccine.

ately wearing gloves, gown, and mask in the presence of the infected person or only had a very brief contact.

Time and the Need for Revaccinations

No data exist that record the percentage of persons in a cohort who remain free from smallpox over time (in years) after immunization against smallpox.[3] Data from relatively small studies describe levels (titers) of vaccine-induced neutralizing antibodies for up to 50 years after vaccination or revaccination (28,29). No data correlate antibody titers to immunity from disease. Without data describing the duration of protection afforded by a single vaccination, the current model does not consider the need for additional revaccinations over time. Thus, the results for this model only apply to the primary vaccinations. The model does not discount risk over-time, although some evidence exists that persons have a high discount rate for personal health issues (30).

Results

When these decision criteria are used for a person in a general population of 280 million, the risk for serious vaccine-related adverse events is greater than the risk for smallpox (Figure 1a). This is true even if the risk for a smallpox attack is 1 in 10. An individual person would therefore decline preexposure vaccination. Only when the population at risk is limited to 9 million and the risk for attack approaches 1 in 10, does the risk for smallpox outweigh the risk for serious vaccine-related adverse events, indicating that the person would accept vaccination (Figure 1a). For a HCW to accept preexposure vaccination, the risk for contact with an infectious case of smallpox must be ≥1 in 100 and the probability of an attack causing 1,000 cases must be >1 in 1,000 (Figure 1b). If the risk for contact drops to 1 in 1,000, then the risk for attack must be >1 in 100 to accept vaccination (Figure 1b). For a member of an investigation team, if the anticipated risk for contact is 1 in 2.5 and the risk for attack is ≥1 in 16,000, then a team member would accept preexposure vaccination (Figure 1c). If the risk for contact drops to 1 in 5, then the risk for attack must increase to ≥1 in 8,000 for the person to accept preexposure vaccination (Figure 1c).

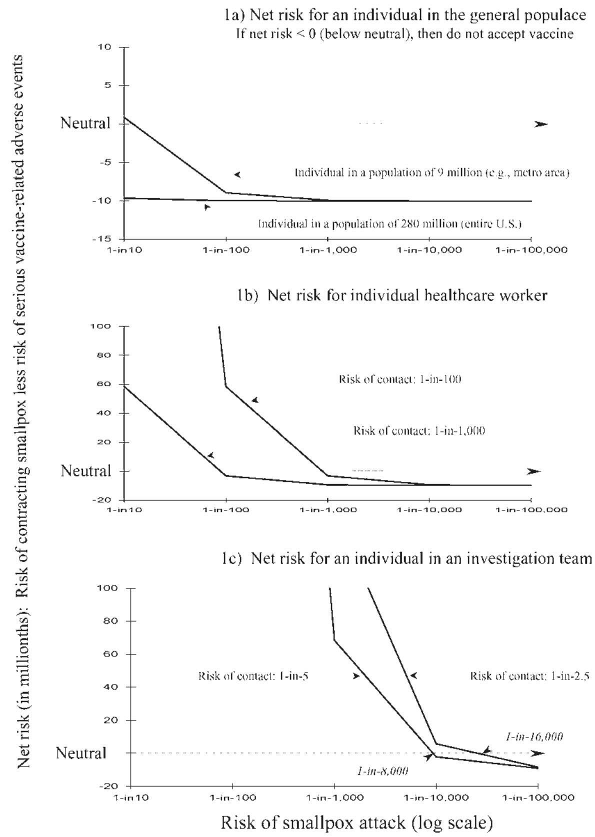

Figure 1. Risk-benefit analyses for individual persons evaluating the risk for smallpox versus the risk for serious smallpox vaccine-related adverse events: three scenarios. If the net risk is >0 (above neutral), then a person will accept preexposure vaccination. If the net risk is <0 (below neutral), then the person would not accept preexposure vaccination. Part a considers a person who is either a member of a population of 9 million, representing a metropolitan area assumed to be the sole target of a smallpox attack and the entire U.S. population of approximately 280 million. In part b, the risk for contact by an individual healthcare worker is a function of probability of contact x probability of transmission (P_E x P_T, see Table and text for further details). In part c, investigation team members are assumed to take precautions against transmission (e.g., wear gloves, face masks, and gowns) to reduce risk to 0.4 (no data of the actual reduction in risk due to using such barrier precautions). Threshold values of risk for smallpox attack, when net risk = 0 (neutral), are rounded to the nearest 1,000. All three parts present data calculated on the basis of an attack that initially causes 1,000 cases before detection of the attack. See Table and text for other assumptions.

Sensitivity Analyses

If a member of the general population of 280 million were to equate 1 case of smallpox to 35 cases of serious

[3] In data reported by Rao from Madras, India (Figures 17/1 and 17/3 [23]), among the unvaccinated, approximately 80% of all cases of smallpox occurred in children <10 years of age. A distinct shift in age of the case-patients occurred among the vaccinated, with <10% of cases occurring in children <10 years of age, 19% of cases occurring in children 10-19 years, and 46% of cases occurring in persons 20-30 years of age. Rao did not report at what age most of those vaccinated received vaccine (a likely hypothesis would be before 2 years of age). Further complicating the analysis of such data is the fact that many persons in Madras received more than one smallpox vaccination (Figure 17/5, [23]). A similar age-shift in occurrence of cases among the vaccinated can be discerned from the data reported by Hanna (24) from Liverpool, England, in 1902-03. The data from both Rao (23) and Hanna (26) further indicate that even a 20-year-old vaccination may reduce the severity of disease. The risk for death is markedly reduced 20-30 years postvaccination (23,26,27).

vaccine-related adverse events, they would accept preexposure vaccination only if the risk for a smallpox attack approached 1 in 10 (Figure 2a). However, if the risk for attack is assumed to be 1 in 100, then the person would have to equate 1 case of smallpox to 290 cases of serious vaccine-related adverse events to accept preexposure vaccination (data not shown). If a person assumes both that the initial attack would cause 100,000 cases before detection and that 1 case of smallpox is equivalent to 35 cases of serious vaccine–related adverse events, then the risk for attack would have to be >1 in 1,000 to accept preexposure vaccination (Figure 2b).

Assuming a risk for serious vaccine-related adverse events of 1 in 10,000 (25) and the same values used to produce Figure 1a, a person in a population of 9 million would not accept vaccination even if the risk for attack were 1 in 2. When the same risk for adverse events is used in considering the scenarios evaluated in Figure 2b (100,000 cases before detection, valuation of 1 case smallpox = 35 cases of vaccine-related adverse events), the risk for attack would have to be >8 in 1,000 before accepting vaccination (results not shown).

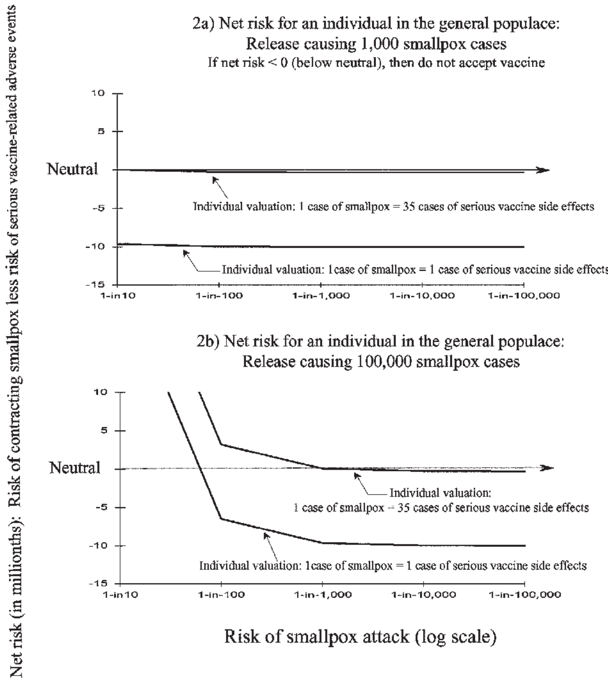

Figure 2. Sensitivity analyses: impact of altering a person's value of a case of smallpox relative to a case of serious smallpox vaccine-related adverse events. If the net risk is >0 (above neutral), then a person will accept preexposure vaccination. If the net risk is <0 (below neutral), then the person would not accept preexposure vaccination. Both parts show the impact of altering a person's valuation of a case of smallpox relative to a case of serious vaccine-related adverse events. Part a shows the net risks for an individual person's considering preexposure smallpox vaccination with an attack causing clinical cases of smallpox to develop in 1,000 persons. Part b shows the net risks for a person when an attack causes clinical cases of smallpox to develop in 100,000 persons (see text for further details).

Revaccination

For a person in a population of 280 million who is considering preexposure revaccination with a risk for serious vaccine-related adverse events of 1 in 1,000,000, even at a 1 in 10 risk for smallpox attack, the net risk is <0, and the decision criteria would indicate not accepting revaccination (scenario assumed 1,000 smallpox cases before discovery of the attack, and setting $P_{Valuation}$ = 1:1). In the same scenario, if $P_{Valuation}$ = 1:35, then the risk for a smallpox attack would have to be >1 in 125 for a person to accept revaccination. For a HCW to accept preexpsoure vaccination, the risk for attack would have to be >1 in 700 (risk for contact = 1-in-1,000; $P_{Valuation}$ = 1:1; revaccination $P_{SideEffect}$ = 1-in-1,000,000). If the HCW assumed that the risk for contact increased to 1 in 100, then the risk for attack would have to be >1 in 7,000 in order to accept revaccination.

Postexposure Vaccination

After uncertain exposure to smallpox (e.g., contact with a person who may or may not be infectious with smallpox), the decision criteria would indicate acceptance of postexposure vaccination if the risk for exposure is thought to be ≥1 in 21,000; the risk for transmission is assumed to be 35%, and efficacy of postexposure vaccine is 60% (Figure 3a). If the risk for transmission is assumed to be 70%, but postexposure vaccine efficacy only 10% (e.g., postexposure vaccination offered several days after potential exposure), vaccination would be accepted only if the risk for exposure is assumed to be ≥1 in 8,000 (Figure 3a). If postexposure vaccine efficacy were set at 98%, and risk for transmission at 70%, then risk for actual exposure to smallpox would have to be ≥1 in 69,000 in order to accept postexposure (data not shown).

For persons who have had a definite exposure to smallpox, the only time that postexposure vaccination would not be accepted is if vaccine efficacy was ≤1%, risk of transmission was ≤1%, and the risk for serious vaccine-related adverse events were ≥1 in 5,000 (Figure 3b). In the same scenario, if the risk for transmission were 30%, postexposure vaccination would accepted even if risk for serious vaccine-related adverse events were 1 in 500 (Figure 3b).

Figures 1 and 2 show that the single most influential variable impacting the net risk for disease, and therefore the decision to accept preexposure vaccination, was the probability of attack of smallpox. For persons in the general population, the second most important variable is the valuation of one case of smallpox relative to cases of serious vaccine-related adverse events ($P_{Valuation}$). For a HCW or a member of an investigation team, the second most

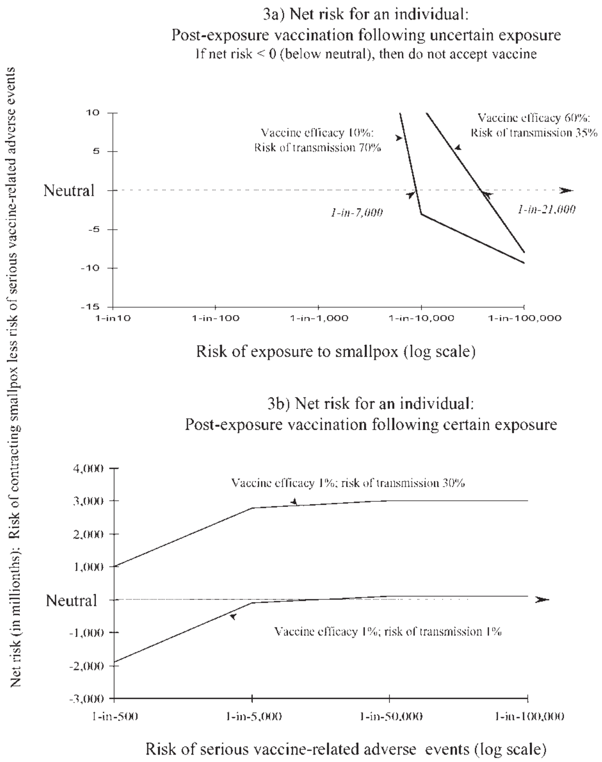

Figure 3. Risk-benefit analyses for persons considering postexposure smallpox vaccination: two scenarios. If the net risk is >0 (above neutral), then the person will accept postexposure vaccination. In the net risk is <0 (below neutral), then the person would not accept postexposure vaccination. Part a shows the net risk for postexposure smallpox vaccination for a person who has been exposed to somebody who may or may not have smallpox (i.e., the exposure is uncertain). Threshold values of risk for exposure to smallpox, when net risk = 0 (neutral), are rounded to the nearest 1,000. Part b shows the net risk for an individual person who has been exposed to a definite smallpox case (see text for further details).

important variable was the risk for contact with a smallpox patient or infectious material.

Conclusions

The model suggests that most persons in the general population would not accept preexposure smallpox vaccination. Increasing the risk for vaccine-related adverse events (e.g., including the risk for eczema vaccinatum and vaccine-related myocarditis orpericarditis) moves all the graph lines in Figures 1 and 2 downward. This supposition increases the likelihood of not accepting preexposure vaccination. These results and conclusions are not unique. In 1971, some argued that the risks for routine childhood smallpox vaccination in the United States outweighed the risks of contracting a case of smallpox (4,31,32). These arguments influenced the 1971 recommendation to stop routine childhood immunization against smallpox in the United States (33). The studies and arguments influencing the decision took an implicit societal perspective, while this study considers the perspective of the individual person.

For an individual healthcare worker, the decision to accept preexposure vaccination hinges almost as much on the assessment of risk for contact (before discovery of attack) as on the assessment of risk of attack. In the mid-Atlantic states of New Jersey, New York, Pennsylvania (New Jersey, New York, and Pennsylvania), approximately 440 general hospitals exist; 83% operate an emergency room (34). These hospitals are staffed by approximately 18,000 full-time equivalents (FTEs) physicians and dentists, 160,000 nurse FTEs (in NY 1 nurse FTE = 1.13 persons), 24,144 trainees and approximately 430,000 "other salaried" staff, for a total staff of approximately 650,000 (34,35). If one assumes that 10% work in the emergency rooms, 65,000 hospital staff in New Jersey, New York, and Pennsylvania are vulnerable to infection before a smallpox attack is detected. Further assume that an attack causes 1,000 smallpox cases confined to the New Jersey, New York, Pennsylvania area. By days 7–8 postinfection, <20% of those will proceed to the prodrome and rash stages (1,2), perhaps causing medical care to be sought. Blendon et al. (13) reported that 52% of survey respondents stated that they would go to their own family doctor if they thought they had smallpox, with 42% stating that they would go to a hospital emergency room. Thus, approximately 100 patients (1,000 x 20% early cases x 50% to hospital) might seek medical care at a hospital in the first 7–9 days after infection.

The healthcare workers in emergency rooms therefore face a risk for exposure to an infectious smallpox patient of change to <1 in 600 (65,000/100). If one assumes a risk for transmission of 70%, the risk of contracting smallpox is almost 1 in 1,000. The many part-time and temporary workers in a hospital further reduces this risk ratio. Even if one patient can potentially infect up to 10 healthcare workers in a hospital setting (36), the risk is still 1 in 65. Note that the risk for exposure is not confined to medical doctors or nurses. Many members of a hospital staff, such as those working in housekeeping and maintenance, are at risk of coming into contact with an infectious patient.

Figure 3a may suggest to some that almost any exposure to a possible case of smallpox, such as coming into contact with a person with an unexplained rash, would warrant immediate postexposure vaccination (e.g., before laboratory confirmation that patient with unknown rash actually had smallpox). However, postexposure vaccination given within 7 days after exposure reduces the risk of a clinical case of smallpox developing to approximately 2% compared with 79% among those never vaccinated (37). If vaccination is delayed up to 10 days postexposure, then the risk for smallpox may be reduced just 22% (from 96% among those never vaccinated to 75% among those vaccinated within 10 days postexposure) (38).[4] A more compelling conclusion from Figure 3a is that if, by day 6 postexposure, the type of exposure cannot be accurately determined and a person could have been exposed to smallpox (i.e., risk for exposure is ≥1 in 21,000), then the person would use the decision criteria to accept postexposure vaccination.

The biggest problem in interpreting the results from the model is understanding how a person will actually value risks and events. Valuing risks depends on understanding probabilities, which are often difficult to explain (41). Even the type of visual aid used to explain risk can make a difference in valuation (42). Merely stating a number (e.g., 1 in 10,000) is often not sufficient. A person's valuation of the risks and benefits of vaccination may include factors not explicitly defined in the model. A person may accept preexposure vaccination, for example, as an attempt to reduce potential risk for smallpox to family and friends and even out of a sense of duty to society in general. The valuation of a case of smallpox relative to a case of serious vaccine-related adverse events is a proxy for valuing a person's contribution to family, friends, and society.

Public health planners and medical care providers should appreciate the extent that an individual acceptance or rejection of smallpox vaccination depends on valuation of risk and benefits. A person's risk aversion is not completely explained by numerical analyses (43,44). A person's valuation of risk depends on a variety of factors, including a sense of control, degree of trust of the source providing the data, the newness of the risk, and even the passage of time (41,45). Note that time and information may not alter the actual risk faced, but a factor such as new information (e.g., reported cases of vaccine-related adverse events) could alter the perception and valuation of risk. Accurately predicting the direction and extent of a change in valuation attributable to, for example, new information may not be possible. Public health officials, however, must always be prepared to explain how the new information alters the risks involved. Explaining a given risk, and how a new development may impact that risk, will likely require more than just a single numerical statement.

[4]In addition to the estimates quoted in the main text, Rao et al. (39) found that successful postexposure vaccination reduced, on average, the rate of smallpox among contacts by approximately 38% (from 48% among unvaccinated to 30% among postexposure vaccinees). Dixon (40) reported that in a group of 59 contacts under 5 years of age ". . . approximately half of those who had a successful vaccination after contact developed disease." The wide variations in reports of the degree of protection afforded by postexposure vaccination are probably due to a number of reasons, including small sample sizes and difficulty in determining when exposure and potential transmission actually took place.

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Toxoplasma gondii Infection in the United States, 1999–2000

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Infection with *Toxoplasma gondii* can lead to congenital and acquired disease, resulting in loss of vision and neurologic illness. We tested sera collected in the National Health and Examination Survey (NHANES) from 1999–2000 for *T. gondii*–specific immunoglobulin G antibodies and compared these results with results from sera obtained in the NHANES III survey (1988–1994). NHANES collects data on a nationally representative sample of the U.S. civilian population. Of 4,234 persons 12–49 years of age in NHANES 1999–2000, 15.8% (age-adjusted, 95% confidence limits [CL] 13.5, 18.1) were antibody positive; among women (n = 2,221) 14.9% (age-adjusted, 95% CL 12.5, 17.4) were antibody positive. *T. gondii* antibody prevalence was higher among non-Hispanic black persons than among non-Hispanic white persons (age-adjusted prevalence 19.2% vs. 12.1%, p = 0.003) and increased with age. No statistically significant differences were found between *T. gondii* antibody prevalence in NHANES 1999–2000, and NHANES III. *T. gondii* antibody prevalence has remained stable over the past 10 years in the United States.

Toxoplasma gondii is a ubiquitous protozoan parasite of warm-blooded animals. However, only members of the cat family (*Felidae*) are definitive hosts for the organism, which is shed in their feces for several weeks after the organism has completed a sexual cycle in their intestinal epithelial cells. Infection in humans generally occurs either by ingesting viable tissue cysts in raw or undercooked meat or by ingesting oocysts shed in the feces of a cat. After acute infection, *T. gondii* continues to exist in tissue cysts in humans, particularly in the muscles and brain. However, in people with immunodeficiencies such as AIDS or malignancies, rupture of cysts results in disease reactivation, including encephalitis or disseminated toxoplasmosis. Immunoglobulin (Ig) G antibodies to *T. gondii* appear early, reach a peak within 6 months after infection, and are detectable for life.

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When a pregnant woman is infected for the first time, and the infection spreads to the fetus, congenital *T. gondii* infection may be clinically apparent in the neonate in the first months of life or later during infancy, childhood, or adolescence (i.e., cause neurologic or eye damage) or may remain subclinical. An estimated 400 to 4,000 cases of congenital toxoplasmosis occur each year in the United States (1). In an analysis of data from a large HIV-infected cohort, toxoplasmosis was found to be the most frequent severe neurologic infection among persons with AIDS in the United States, even after the advent of highly active antiretroviral therapy (2).

The United States Department of Agriculture (USDA) estimates that one half of *T. gondii* infections in the United States are caused by ingestion of raw or undercooked infected meat (3). A community-based study in Maryland, comparing persons who did not eat meat with those who did eat meat, supports the USDA estimate (4). In 1999, Mead and colleagues estimated that of the 750 deaths caused by toxoplasmosis each year, one half were the result of eating raw or undercooked meat, making toxoplasmosis the third leading cause of foodborne deaths (5).

To present the prevalence of infection in the U.S. population, we report the *Toxoplasma*-specific IgG results of the National Health and Nutrition Survey (NHANES) conducted in 1999–2000 and compare the prevalence of *Toxoplasma* IgG antibody seropositivity during these years to the prevalence observed previously in NHANES III 1988–1994. In the NHANES III national probability sample, 22.5% of 17,658 persons $\geq$12 years of age had *Toxoplasma*-specific IgG antibodies, indicating that they had been infected with the organism (published prevalence was age-adjusted to the 1980 U.S. population; prevalence for same population age-adjusted to the 2000 U.S. population is 23.6%) (6).

Methods

NHANES Samples

Beginning in 1999, NHANES became a continual survey. Each survey year is based on a nationally representative sample of the U.S. civilian noninstitutionalized population, selected with a stratified, multistage, probability cluster design. Data are collected on health measures and conditions through household interviews, standardized physical examinations, and blood samples obtained at mobile examination centers. The procedures followed to select the sample and conduct the interviews and examinations are similar to those of previous NHANES surveys (6). The continual NHANES is released in 2-year groupings (cycles). Two or more years of data are necessary to have adequate sample sizes for subgroup analyses. This report is based on the first 2 years of the continual NHANES (1999–2000).

Serum samples were available for testing for *T. gondii* antibodies from a nationally representative sample of persons 6–49 years of age in NHANES 1999–2000 and from persons $\geq$12 years of age in NHANES III. To compare NHANES 1999–2000 with NHANES III, the principal analyses are limited to the overlapping age groups (i.e., 12–49 years of age) and stratified on variables previously examined in NHANES III (6). Age was grouped as 12–19, 20–29, 30–39, and 40–49 years. In NHANES III, serum specimens were also available from a limited number of children 6–11 years of age (n = 1,819, 48% of children sampled in this age range). Although the NHANES III data in this 6–11 year age group cannot be considered nationally representative because of the low response rate, we present the previously calculated prevalence (6) for this group in our results because these are the only U.S. data available for this age.

Race/ethnicity was defined as self-reported non-Hispanic white, non-Hispanic black, or Mexican American (in NHANES III and NHANES 1999–2000, oversampling of Mexican Americans was conducted to ensure adequate sample size for this group). In NHANES 1999–2000, the race/ethnicity variable used was the one most consistent with the NHANES III race/ethnicity coding. The NHANES 1999–2000 sample size was not sufficient to stratify by other racial and ethnic groups; however, these groups were included in the estimates given for the total study population.

Informed consent was obtained from patients or their parents or guardians, and human subjects review guidelines of the U.S. Department of Health and Human Services were followed in the conduct of this research.

Laboratory Testing

NHANES 1999–2000 specimens were tested by using the Platelia Toxo-G enzyme immunoassay kit (BioRad, Hercules, CA), according to the manufacturer's instructions. Results were reported in international units (IU); samples with $\geq$10 IU were considered positive for *T. gondii* IgG antibodies. NHANES III serum specimens had been tested with the same kit, however >6 IU was used as a cutoff for seropositivity. As a result of minor changes in the kit, the company changed the IU cutoff value for kits used to test the 1999–2000 sera. However, test positivity should be considered to be equivalent for both studies, regardless of the cutoff values.

Statistical Analysis

Prevalence estimates were weighted to represent the U.S. population, to account for oversampling in specific demographic subgroups, and to account for nonresponses to the household survey and to the physical examination. Estimates and standard errors were calculated by using SUDAAN (7). Standard errors for NHANES 1999–2000 were estimated by means of the delete 1 jackknife (JK1) method (8). In previous NHANES surveys, the Taylor series linearization method was used to estimate standard errors. These standard errors account for the sample weights and complex sample design. Prevalence estimates were age-adjusted by the direct method to the 2000 U.S. population for both NHANES III and NHANES 1999–2000 when seroprevalence was compared across population subgroups. Ninety-five percent confidence intervals were calculated by using a t-statistic; p values testing the significance of the difference in prevalence between NHANES III and NHANES 1999–2000 were obtained by using a t-statistic with the combined standard error.

Results

Of the 4,875 persons 12–49 years of age who were selected for NHANES 1999–2000, a total of 4,602 (94.4%) persons were interviewed and underwent physician examination; of these, 4,234 persons (86.9% of those selected) had serum specimens tested for *T. gondii* antibodies. In NHANES 1999–2000, the percentage of those tested for *T. gondii* IgG antibodies among those examined did not vary by race/ethnicity, sex, or country of birth. Some variability existed, but no consistent trend, with age in the percentage of persons with sera tested among those examined (range 91% to 94%). Of the 4,234 persons tested for *T. gondii* IgG antibodies, 638 (15.8%, age-adjusted, 95% confidence limits [CL] 13.5, 18.1) were antibody positive. Among women (n=2,221), 14.9% (age-adjusted, 95% CL, 12.5, 17.4) were antibody positive. *T. gondii* antibody prevalence for men was similar to that for women (age-adjusted, 16.7% vs. 14.9%, respectively, p=0.28), higher among non-Hispanic blacks than among non-Hispanic whites (age-adjusted, 19.2% vs. 12.1%, p=0.003), and higher as age increased (Table). The *T. gondii* antibody prevalence was also higher in Mexican Americans than in non-Hispanic whites, but the difference was not statistically significant (16.8% vs. 12.1%, p=0.051). In NHANES III, the age-adjusted seropreva-

Table. Comparison of *Toxoplasma gondii* immunoglobulin G antibody seroprevalence in NHANES 1999–2000 and NHANES III (1988–1994)[a,b,c]

	NHANES 1999–2000			NHANES III		
	N[d]	Prevalence	95% CL	N[d]	Prevalence	95% CL
Total	4,234	15.8	13.5, 18.1	11,132	16.0	14.5, 17.5
Sex						
Male	2,013	16.7	13.6, 19.9	5,144	16.7	14.8, 18.6
Female	2,221	14.9	12.5, 17.4	5,988	15.3	13.5, 17.0
Race/ethnicity						
Non-Hispanic white	1,293	12.1	9.9, 14.4	3,304	14.3	12.5, 16.2
Non-Hispanic black	1,027	19.2	14.8, 23.6	3,674	18.0	16.1, 19.8
Mexican American	1,553	16.8	12.4, 21.1	3,661	18.3	16.7, 20.0
Age group						
12–19	2,105	9.3	6.4, 12.1	2,749	8.5	6.4, 10.5
20–29	735	13.4	10.1, 16.7	3,100	15.2	12.1, 18.3
30–39	726	18.1	14.7, 21.5	2,960	16.1	14.6, 17.6
40–49	668	20.4	15.7, 25.0	2,323	22.2	19.4, 25.0
Country of birth						
United States	3,211	12.2	10.0, 14.3	8,606	14.1	12.7, 15.5
Non-U.S.	995	32.8	27.3, 38.3	2,493	27.9	24.1, 31.7

[a]NHANES, National Health and Examination Survey.
[b]Sex, race/ethnicity, and total values are age-adjusted to the 2000 census estimated population, using the four age categories shown in the table.
[c]No statistically significant differences (p>0.05, t-statistic) existed between NHANES 1999–2000 and NHANES III across any subgroup in the table.
[d]Totals for the race/ethnicity or country of birth categories do not add up to the total number because of an "other" category for race/ethnicity (not shown) or because persons did not provide a response to country of birth questions.

lence was similar for men and women and higher in Mexican Americans than in non-Hispanic whites in the 12–49 year age range.

No significant differences were found between NHANES 1999–2000 and NHANES III *T. gondii* antibody prevalences overall or in any of the sex, race, or age categories (Table, comparing values horizontally by rows). In NHANES 1999–2000, children 6–11 years of age had a *T. gondii* antibody prevalence of 8.0% (95% CL 4.5, 11.5, N = 855) (data not shown in table). In NHANES III, the antibody prevalence for children 6–11 years of age was 5.2% (6), however, as noted in Methods, this estimate may be subject to nonresponse error (data not shown in table).

The *T. gondii* antibody prevalence was higher in persons born outside the United States than in U.S.-born persons for both NHANES 1999–2000 and NHANES III (age-adjusted, 32.8% vs. 12.2% and 27.9% vs. 14.1%, respectively, Table), but among persons born outside the United States seroprevalence did not differ significantly between NHANES 1999–2000 and NHANES III (p>0.05). In addition, the percentage of persons that were born outside the United States was not significantly different in NHANES 1999–2000 (16.3%, 95% CL 11.8%, 20.7%) compared with the percentage of persons born outside the United States in NHANES III (13.3%, 95% CL 10.9%, 15.7%) (p > 0.05).

Discussion

We found an overall *T. gondii* IgG antibody prevalence of 15.8% among persons 12–49 years of age in 1999–2000, indicating that approximately 1 in 6 persons in this age group was infected with *T. gondii*. No significant changes in *T. gondii* seroprevalence occurred between 1988–1994 and 1999–2000 for the U.S. population as a whole or for any of the subgroups we examined. We had speculated that changes in meat production with lower levels of *T. gondii* in meat (9) might result in a reduction in the prevalence of *T. gondii* infection in the population. Perhaps the time was not sufficient for changes in meat production and consumption habits to have had an impact, or perhaps the expected declines in *T. gondii* infection occurred before NHANES III. The prevalence of *T. gondii* infection declined in U.S. military recruits, when 1965 data were compared with 1989 data (10) and in countries in Europe, such as France and Belgium, during similar periods (11).

Predicting future trends in *T. gondii* prevalence in the United States is difficult because we do not have a national estimate of what proportion of *T. gondii* infections are attributable to undercooked meat exposure or to cat feces, soil, or water exposure. A large European case-control study that examined these factors showed that undercooked meat accounted for the largest portion of risk for *T. gondii* infection (30%–63%, depending on location) (12). However, until researchers examine the risk factors for *T. gondii* infection in a case-control study throughout the United States, the most important U.S. risk factors and how to best focus preventive education messages will not be determined.

In NHANES 1999–2000, the *T. gondii* antibody prevalence was higher among non-Hispanic black persons than non-Hispanic white persons. This finding may reflect immigration patterns from countries with higher rates of *T.*

gondii infection or soil exposure and culinary practices among these different populations. The seroprevalence among persons born outside the United States tended to be higher in NHANES 1999–2000 than in NHANES III, and the percentage of persons born outside the United States tended to be higher in NHANES 1999–2000 than NHANES III, but these findings were not statistically significant. Clearly, in both NHANES III and NHANES 1999–2000 the seroprevalence is higher among persons not born in the United States than in U.S.-born persons. The NHANES 1999–2000 sample population is not large enough to stratify racial/ethnic groups by foreign-birth status and obtain accurate estimates; however, in a multivariate analysis reported previously that used NHANES III data (6), being born outside the United States was a significant risk factor for *T. gondii* seropositivity. However, race/ethnicity did not increase risk (using white non-Hispanic persons as the reference group).

NHANES gives representative estimates of prevalence for the U.S. population but is not designed to evaluate local *T. gondii* prevalence levels. Studies have indicated that *T. gondii* prevalence varies greatly in the United States (10,13,14); this local variation is most likely related to culinary practices, the ability of oocysts to survive in different climates, and the levels of immigration from areas of the world in which *T. gondii* infection is highly endemic. Nevertheless, NHANES produces useful surveillance data for tracking *T. gondii* prevalence over time in the United States. We will continue to monitor trends in this nationally representative survey.

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Toxoplasma gondii and Schizophrenia

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Recent epidemiologic studies indicate that infectious agents may contribute to some cases of schizophrenia. In animals, infection with *Toxoplasma gondii* can alter behavior and neurotransmitter function. In humans, acute infection with *T. gondii* can produce psychotic symptoms similar to those displayed by persons with schizophrenia. Since 1953, a total of 19 studies of *T. gondii* antibodies in persons with schizophrenia and other severe psychiatric disorders and in controls have been reported; 18 reported a higher percentage of antibodies in the affected persons; in 11 studies the difference was statistically significant. Two other studies found that exposure to cats in childhood was a risk factor for the development of schizophrenia. Some medications used to treat schizophrenia inhibit the replication of *T. gondii* in cell culture. Establishing the role of *T. gondii* in the etiopathogenesis of schizophrenia might lead to new medications for its prevention and treatment.

Schizophrenia is a pervasive neuropsychiatric disease of uncertain cause that affects approximately 1% of the adult population in the United States and Europe. An increased occurrence of schizophrenia in family members of affected persons suggests that genetic factors play a role in its etiology, and some candidate predisposing genes have been identified. Environmental factors are also important. Epidemiologic studies, for example, have established that winter-spring birth, urban birth, and perinatal and postnatal infection are all risk factors for the disease developing in later life. These studies have rekindled an interest in the role of infectious agents in schizophrenia, a concept first proposed in 1896 (1). This review focuses on evidence specifically linking infection with *Toxoplasma gondii* to the etiology of some cases of schizophrenia.

T. gondii is an intracellular parasite in the phylum Apicomplexa. Its life cycle can be completed only in cats and other felids, which are the definitive hosts. However, *T. gondii* also infects a wide variety of intermediate hosts, including humans. In many mammals, *T. gondii* is known to be an important cause of abortions and stillbirths and to selectively infect muscle and brain tissue. A variety of neurologic symptoms, including incoordination, tremors, head-shaking, and seizures, have been described in sheep, pigs, cattle, rabbits, and monkeys infected with *T. gondii* (2).

Humans may become infected by contact with cat feces or by eating undercooked meat. The importance of these modes of transmission may vary in different populations (3). Individual response to *Toxoplasma* infection is determined by immune status, timing of infection, and the genetic composition of the host and the organism (4).

Toxoplasma organisms have also been shown to impair learning and memory in mice (5) and to produce behavioral changes in both mice and rats. Of special interest are studies showing that *Toxoplasma*-infected rats become less neophobic, leading to the diminution of their natural aversion to the odor of cats (6). These behavioral changes increase the chances that the rat will be eaten by a cat, thus enabling *Toxoplasma* to complete its life cycle, an example of evolutionarily driven manipulation of host behavior by the parasite.

In humans, *Toxoplasma* is an important cause of abortions and stillbirths after primary infection in pregnant women. The organism can also cross the placenta and infect the fetus. The symptoms of congenital toxoplasmosis include abnormal changes in head size (hydrocephaly or microcephaly), intracranial calcifications, deafness, seizures, cerebral palsy, damage to the retina, and mental retardation. Some sequelae of congenital toxoplasmosis are not apparent at birth and may not become apparent until the second or third decade of life. Hydrocephalus (7), increased ventricular size (8), and cognitive impairment (9) have also been noted in some persons with schizophrenia and other forms of psychosis.

Some cases of acute toxoplasmosis in adults are associated with psychiatric symptoms such as delusions and hallucinations. A review of 114 cases of acquired toxoplasmosis noted that "psychiatric disturbances were very frequent" in 24 of the case-patients (10). Case reports describe a 22-year-old woman who exhibited paranoid and

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bizarre delusions ("she said she had no veins in her arms and legs"), disorganized speech, and flattened affect; a 32-year-old woman who had auditory and visual hallucinations; and a 34-year-old woman who experienced auditory hallucinations and a thought disorder (11). Schizophrenia was first diagnosed in all three patients, but later neurologic symptoms developed, which led to the correct diagnosis of *Toxoplasma* encephalitis.

Psychiatric manifestations of *T. gondii* are also prominent in immunocompromised persons with AIDS in whom latent infections have become reactivated. Reviews of such AIDS cases with toxoplasmosis have indicated that altered mental status may occur in as many as 60% of patients and that the symptoms may include delusions, auditory hallucinations, and thought disorders (12).

Additional studies have documented that persons with serologic evidence of *Toxoplasma* infection have evidence of psychiatric changes in the absence of a history of clinically apparent *Toxoplasma* infection. Studies in which personality questionnaires have been administered to healthy adults have indicated that serum antibodies to *T. gondii* are associated with alterations in behavior and psychomotor skills (13). Seropositivity to *Toxoplasma* has also been associated with "lack of energy or tiredness" in schoolchildren (14). In view of these findings, we decided to carry out serologic and other studies and to survey the literature for possible additional links between *Toxoplasma* infection and schizophrenia.

Serologic Studies of Patients with Schizophrenia

Studies Before 1980

In the course of doing our studies, we discovered that much research had been published in languages other than English and was not listed on searchable databases. Through direct contact with authors and by obtaining references listed on their papers, we identified 13 relevant studies published between 1953 and 1979 (15–27), as listed on the Table. Some publication bias is likely, since negative studies are less likely to have been submitted or published.

The 13 studies used a variety of immunologic methods for measuring antibodies, including the Sabin Feldman dye test, skin tests, and complement fixation (CF). One study used a test in which an alkaloid from *T. gondii* caused a tropical fish, *Lebistes reticulatus*, to change color (19). Some of the studies compared the relative efficacy of two different tests. Most of the studies defined *Toxoplasma*-positive results as the presence of a skin reaction or antibodies above a certain titer but often without specifying the precise details of the method; thus, comparing the older studies with each other was not possible. Most of these studies also did not specify what diagnostic criteria were used for schizophrenia, but since at least 12 of them used inpatients, the patients likely had a severe psychiatric disorder. Similarly, most of the studies did not specify the origin of their control group other than saying such things as "681 healthy persons working or studying in the city of Gdansk" (15).

Despite these limitations, 12 of the 13 studies found that the patient group had a higher percentage of antibodies to *Toxoplasma* than the control group. In eight of the studies, the increase was statistically significant by chi square at the level of $p < 0.05$. In the two largest studies, Kozar (15) in Poland reported antibodies in 495 (52%) of 961 psychiatric inpatients compared with 170 (25%) of 681 controls, and Roch and Varela (25) in Mexico found antibodies in 836 (86%) of 973 patients with schizophrenia compared with finding antibodies in 30% of the general population.

Studies Since 1999

We identified no studies that were done between 1979 and 1999. Since that time, six studies have been carried out, including our own (28–32). All used enzyme immunoassay methods for measuring antibodies to *Toxoplasma.* All of the studies also used modern diagnostic criteria for schizophrenia; three studies included patients with chronic disease, and three included patients who were in the first episode of the disease. All of the studies identified their control groups, and some attempts were made to match them to the patient groups.

The results of these studies are summarized in the Table. In all of the studies, the patients had more antibodies to *Toxoplasma* than the control groups, and in the three studies, carried out in China and Germany, of patients who were having their first-episode of schizophrenia, the differences were statistically significant. One of the first-episode studies, carried out in Cologne by Leweke et al. (32), divided the first-episode patients into those who had never received antipsychotic treatment and those who had received some treatment. The antibody levels for the treated group were intermediate between the levels of the never-treated group and those of the control group, suggesting that antipsychotic medication may have decreased the antibody levels. This conclusion is supported by a study that indicated that some antipsychotic medications inhibit the growth of *T. gondii* in cell culture (33).

The Leweke et al. study also collected cerebrospinal fluid (CSF) from the first-episode patients. The level of *Toxoplasma* antibody in the CSF of untreated patients was significantly higher than the normal controls ($p < 0.0001$) (32). Treated first-episode patients had CSF antibody levels intermediate between those of the untreated patients and the controls, just as was found for the sera.

In addition to these studies on adults with schizophrenia, a study was also conducted by analyzing serum samples from pregnant women, obtained shortly before deliv-

Table. Toxoplasmosis antibody studies of psychiatric patients

Y	Author and country	Test used	Patients	Controls	% Patients antibody positive	% Controls antibody positive	p value chi square
Before 1980							
1953	Kozar (15) Poland	Skin test	Psychiatric inpatients, all diagnoses	Healthy persons, ages 18–60	52 (495/961)	25 (170/681)	<0.0001
1956	Vojtechovská et al. (16) Czechoslovakia	Skin test	Inpatients with "psychosis"	General population	59 (68/116)	30 (not specified)	<0.0001
1956	Wende (17) East Germany	Dye test	Inpatients with schizophrenia	Inpatients with neurologic disorders	8 (3/38)	5 (24/520)	0.418
1957	Jirovec et al. (18) Czechoslovakia	Skin test	Inpatients with schizophrenia	Normal population	48 (238/501)	29 (286/970)	<0.0001
1958	Buentello (19) Mexico	Color change in fish	Inpatients with schizophrenia	Normal subjects	69 (29/42)	0 (0/60)	<0.0001
1958	Caglieris (20) Italy	Dye test	Inpatients with schizophrenia	Normal subjects	21 (13/61)	15 (12/81)	0.376
1961	Cook & Derrick (21) Australia	Dye test C.F.	Inpatients with schizophrenia	General population	36 (19/53) ≥1:16 11 (6/53) ≥1:4	24 (182/760) 13 (99/760)	0.053 0.840
1962	Yegerov et al. (22) Russia	Skin test C.F.	Inpatients with schizophrenia	Hospital employees	19 (7/37) 32 (12/37)	4 (1/25) 28 (7/25)	0.124 0.784
1962	Avlavidov (23) Bulgaria	Skin test C.F.	Psychiatric inpatients, not specified	Female surgical patients	26 (5/19) 21 (3/14)	3 (1/35) 9 (3/34)	0.017 0.339
1966	Berengo et al. (24) Italy	Dye test	Inpatients with schizophrenia	General population	14 (76/560)	4 (49/1200)	<0.0001
1966	Roch & Varela (25) Mexico	Dye test	Schizophrenia, hospital status not specified	General population	86 (836/973)	30 (4,411/14,689)	<0.0001
1968	Garrido & Redondo (26) Spain	C.F.	Inpatients with schizophrenia	General population	44 (17/39)	29 (147/500)	0.072
1979	Garcia (27) Cuba	Skin test	Psychiatric inpatients	Normal persons	60 (60/100)	30 (30/100)	<0.0001
Since 1999							
1999	Qiuying et al. (28) China	EIA	Inpatients with schizophrenia	Normal persons from same region for routine physicals	14 (22/152)	10 (41/396)	0.181
2001	Gu et al. (29) China	EIA	First-episode schizophrenia	Normal controls matched for age, sex, birthplace	33 (45/135)	9 (4/43)	0.002
2001	Yolken et al. (30) Germany	EIA: IgG or IgM	First-episode schizophrenia	Normal controls matched for age, sex, SES	42 (16/38)	11 (3/27)	0.007
2002	Boronow et al. (31) United States	EIA	Outpatients with schizophrenia	Normal controls matched for age	12 (28/229)	7 (7/100)	0.147
2003	Leweke et al. (32) Germany	EIA	First-episode schizophrenia, never treated	Normal controls matched for age, sex, SES	36 (13/36)	14 (10/73)	<0.007
2003	Torrey & Yolken (unpub. data) Ireland	EIA	Inpatients with schizophrenia	Hospital employees	60 (31/52)	45 (9/20)	0.299

[a]C.F., complement fixation; EIA, enzyme immunoassay; Ig, immunoglobulin; SES, socioeconomic status.

ery, who gave birth to children in whom schizophrenia or other psychoses developed. Preliminary analysis indicates an increased rate of immunoglobulin (Ig) M (but not IgG) class antibodies to *Toxoplasma gondii* in mothers with infants in whom schizophrenia developed later, suggesting that the mothers were experiencing an active infection or that they had persistent IgM antibodies, as described in other studies. Increased levels of IgM antibodies were not found to other perinatal pathogens such as rubella virus or cytomegalovirus (34).

Discussion

Multiple studies have demonstrated that the brains of persons with schizophrenia show structural and functional changes and that these exist even in patients who have never been treated with antipsychotic medications (35). Thus, schizophrenia, like multiple sclerosis and Parkinson's disease, is a chronic disease of the central nervous system; as with other such diseases, infectious agents should be considered as possible etiologic agents, perhaps in persons who also have an increased genetic susceptibility.

T. gondii is of special interest because of its known affinity for brain tissue and its capacity for long-term infection starting in early life. The effect of *Toxoplasma* infection on any given person may differ, depending on such factors as individual genetic predisposition, the state of the immune system, the dose, the virulence of the infecting strain, the timing (e.g., infections in the first trimester of pregnancy differ from those in the third trimester; prenatal and postnatal infections differ; etc.), and the part of the brain affected.

If *Toxoplasma* is involved in the etiology of schizophrenia, however, its synergy with genes may determine the person's brain development, immune response to infections, and response to other infectious agents. The fact that *T. gondii* has been shown to activate retroviruses in animal model systems may be relevant (36). This property is consistent with the recent finding that many persons with schizophrenia exhibit increased retroviral activation within their central nervous systems (37).

Numerous studies indicate that, although the symptoms of schizophrenia generally do not manifest until late adolescence or early adulthood, the disease process has its origins in earlier stages of brain development. The ability of *Toxoplasma* organisms to infect the perinatal brain is thus consistent with this aspect of schizophrenia pathogenesis. However, prospective studies also support a possible role of postnatal infections in some cases of schizophrenia (38). The potential effects of the transmission of *Toxoplasma* in early childhood or later in life should thus be considered.

Epidemiologically, two studies have reported that adults who have schizophrenia or bipolar disorder had a greater exposure to cats in childhood. In one study, 84 (51%) of the 165 affected versus 65 (38%) of the 165 matched controls had owned a house cat in childhood ($p = 0.02$) (39). In the other study, 136 (52%) of the 262 affected versus 219 (42%) of the 522 matched controls owned a cat between birth and age 13 (odds ratio 1.53; $p < 0.007$) (40). Whether any geographic association exists between the prevalence of toxoplasmosis and the prevalence of schizophrenia is unknown. France, which has a high prevalence of *Toxoplasma*-infected persons, was reported to have first-admission rates for schizophrenia approximately 50% higher than those in England (41). Ireland also has a high rate of *Toxoplasma*-infected persons in rural areas (42), confirmed by the high rate of infection in hospital personnel in our own study. The area of our study in Ireland has also been reported to have a high prevalence of schizophrenia (43).

Neuropathologically, studies of *T. gondii* in cell culture have shown that glial cells, especially astrocytes, are selectively affected (44,45). Postmortem studies of schizophrenic brains have also reported many glial abnormalities (46), including decreased numbers of astrocytes (47). Similarly, animal studies of *Toxoplasma* infections have demonstrated that this organism affects levels of dopamine, norepinephrine, and other neurotransmitters, which are well known to be affected in persons with schizophrenia.

Few data exist concerning the clinical correlates of *Toxoplasma* infection in persons with schizophrenia. A recent study found that persons with schizophrenia who have serologic evidence of *Toxoplasma* infection have increased levels of cognitive impairment compared to age-matched *Toxoplasma*-seronegative patients with similar degrees of psychotic symptoms (31). Additional studies are needed on the possible associations between *Toxoplasma* infections and the symptoms or clinical course of schizophrenia and other psychiatric diseases.

One limitation of studies of *Toxoplasma* infection and schizophrenia is that one cannot conclusively rule out disease-related differential exposure to the organism. Thus, hospitalized patients may be fed undercooked meat, thereby increasing their seropositivity. Alternatively, the authors of one of the studies speculated that the increased patient seropositivity might have been because the patients worked in the hospital gardens, which were also frequented by cats (21). The possible effects of hospitalization, altered behavior, or other artifactual factors on seropositivity can be minimized by the analysis of persons with the recent onset of symptoms, as three studies described above have done.

Studies are ongoing in attempts to better define the relationship of *Toxoplasma* infection to schizophrenia. An initial study of the orbital frontal cortex of 14 persons with schizophrenia (48), in which primers to *T. gondii* were used, did not detect sequences. Studies should also include organisms such as *Neospora caninum* and *Hammondi hammondi,* which are closely related to *T. gondii* and which cross-react serologically (49); *N. caninum* has been detected in human specimens in our laboratory and by others (50). The use of organism-specific antigens generated from molecular cloning and the use of stage-specific antibodies should help elucidate both the specificity and the timing of the infection.

Finally, clinical trials are under way of antimicrobial drugs with anti-*Toxoplasma* activity, such as trimethoprim-sulfamethoxazole and azithromycin, as adjunctive treatment for persons with schizophrenia in double-blind trials. These studies may lead to new methods for the treatment of schizophrenia and other psychiatric disorders that may be associated with *Toxoplasma* and related organisms.

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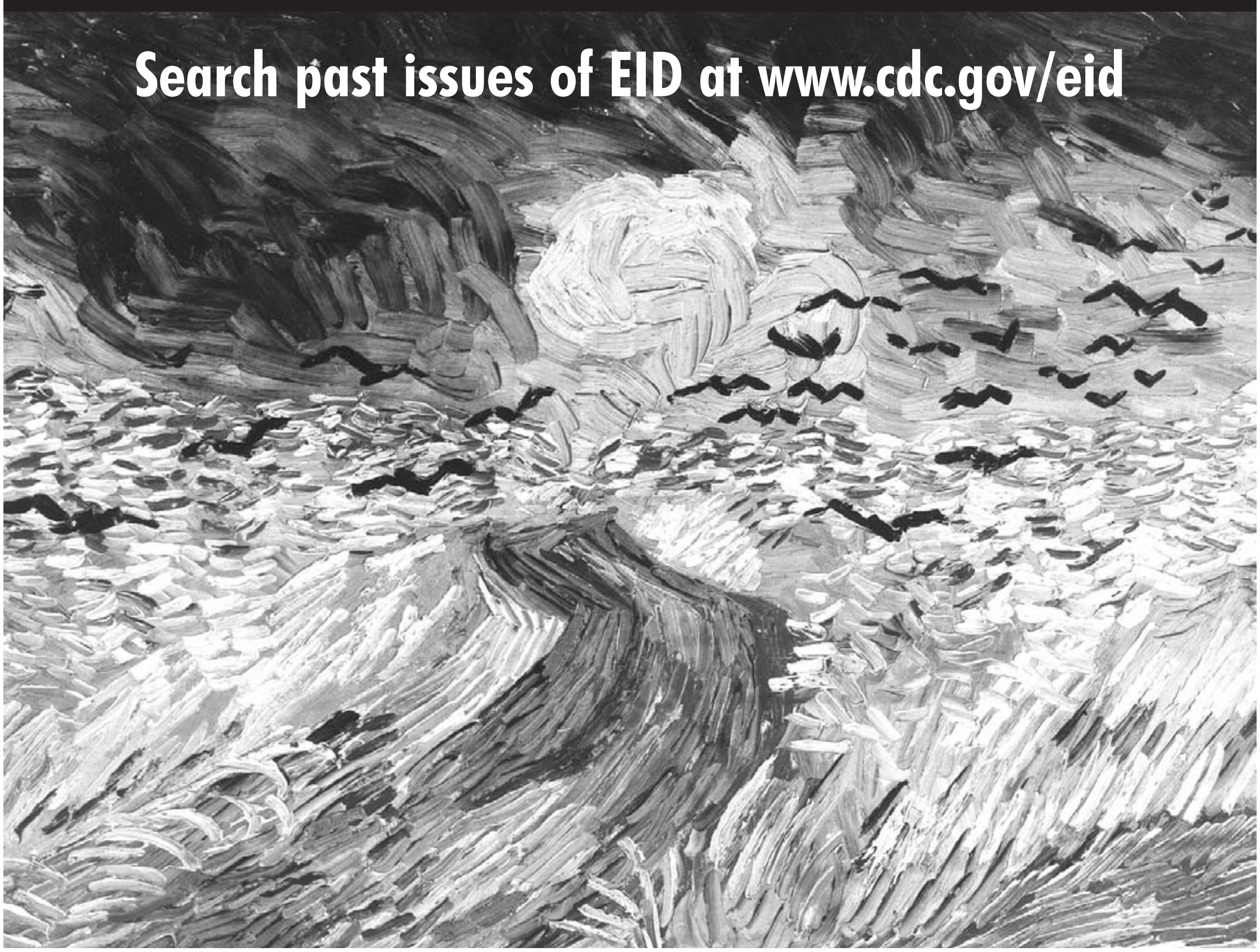

Vincent van Gogh (1853-1890) "Crows in the Wheatfields" 1890. Oil on canvas, 50.35 cm x 103 cm.
Amsterdam, Van Gogh Museum (Vincent van Gogh Foundation)

Coronavirus-positive Nasopharyngeal Aspirate as Predictor for Severe Acute Respiratory Syndrome Mortality

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Severe acute respiratory syndrome (SARS) has caused a major epidemic worldwide. A novel coronavirus is deemed to be the causative agent. Early diagnosis can be made with reverse transcriptase-polymerase chain reaction (RT-PCR) of nasopharyngeal aspirate samples. We compared symptoms of 156 SARS-positive and 62 SARS-negative patients in Hong Kong; SARS was confirmed by RT-PCR. The RT-PCR–positive patients had significantly more shortness of breath, a lower lymphocyte count, and a lower lactate dehydrogenase level; they were also more likely to have bilateral and multifocal chest radiograph involvement, to be admitted to intensive care, to need mechanical ventilation, and to have higher mortality rates. By multivariate analysis, positive RT-PCR on nasopharyngeal aspirate samples was an independent predictor of death within 30 days.

Severe acute respiratory syndrome (SARS) is an emerging infectious disease worldwide. By May 28, 2003, a total of 745 patients had died of SARS and 8,240 persons were infected. At the same time, 270 patients had died of SARS and 1,730 were diagnosed in Hong Kong. As defined by the Centers for Disease Control and Prevention (CDC), a suspected SARS patient is a person with a temperature >38°C; clinical findings such as cough, shortness of breath, and difficulty breathing, together with history of travel to an area with documented local transmission or close contact with a suspected SARS patient within 10 days of symptoms onset. A probable SARS case also requires radiologic evidence of pneumonia or respiratory distress syndrome or autopsy findings consistent with pneumonia or respiratory distress syndrome without an identifiable cause (1). Because of this nonspecific definition, many non-SARS patients may be mislabeled as having SARS. The discovery of coronavirus as the causative agent and the establishment of laboratory tests for coronavirus have aided the research direction. However, these tests only act as supplementary aids to the diagnosis of suspected and probable cases of SARS. The diagnostic tools for coronavirus infection include reverse transcriptase-polymerase chain reaction (RT-PCR), serologic testing, electron microscopy, and viral culture. A fourfold increase in paired serologic test results suggest highest sensitivity and is regarded as the standard criterion for diagnosis. Studies have shown that antibodies against coronavirus are usually present 14–21 days after onset of symptoms. Electron microscopy and viral culture for coronavirus are specific, but the sensitivity is low. The sensitivity of the RT-PCR for coronavirus in nasopharyngeal aspirate (NPA) samples ranges from 32% to 50% at the beginning of an illness and in stool samples is 97% at a mean of 14.2 days (2,3). The variation in sensitivity makes it difficult for the RT-PCR to be the standard criterion for diagnosis. Though the sensitivity is less than perfect, the assay can be used as a tool for early diagnosis. Until now, no data existed regarding the clinical course and outcome of SARS patients with NPA samples that were positive or negative for coronavirus by RT-PCR. We compared the epidemiologic, clinical, laboratory, and radiologic differences between RT-PCR–positive SARS and RT-PCR–negative SARS samples. We also looked for possible microbiologic evidence of coronavirus infection in RT-PCR–negative patients.

Methods

Patients

Two hundred sixty-seven patients fulfilling CDC case definition for suspected or probable SARS were admitted to the isolation wards of the Princess Margaret Hospital from February 26, 2003, to March 31, 2003. RT-PCR on

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NPA became available to us in mid-March. We included in our study 218 patients who had nasopharyngeal RT-PCR performed at illness onset.

Investigation

Routine hematologic, biochemical, and microbiologic tests were performed for all patients. NPA samples were examined by rapid immunofluorescence antigen detection methods for viral cell culture and for common respiratory virus, including influenza viruses A and B; adenovirus; respiratory syncytial virus; and parainfluenza virus types 1, 2, and 3. Sputum samples were screened for bacterial and mycobacterial infection by conventional microscopic identification (Gram staining and acid-fast staining) and culture methods (blood, chocolate, MacConkey, and Löwenstein-Jensen media). Serologic testing for *Mycoplasma pneumoniae*, *Chlamydia pneumoniae,* and *C. psittaci* was performed. Urinary antigen detection tests were used to detect *Legionella pneumophila* and *Streptococcus pneumoniae* in some patients. Paired serum samples were taken 10–14 days apart to assess the serologic response to coronavirus by immunofluorescence assay (IFA). All chest radiographs were classified according to their laterality and extent of involvement.

Qualitative RT-PCR Testing

The NPA sample collected from patients was added into a sterile vial containing 2 mL of viral transport medium and then transported on ice (4°C) to the Public Health Laboratory Centre, Government Virology Unit (GVU) of Hong Kong. Total RNA from 140 µL of each NPA sample was extracted by a QIAamp viral RNA Mini kit (QIAGEN, Valencia, CA), as instructed by the manufacturer and eluted in 60 µL of buffer. A total of 4.2 µL of eluted RNA was reverse-transcribed with use of reverse transcriptase (Applied Biosystem, Foster City, CA) in a 20-µL reaction containing 2.5 µM (final concentration) of random hexamer. The mixture was incubated at room temperature for 10 min and then at 42°C for 15 min. The reaction was stopped at 95°C for 5 min and then chilled in ice. The primers used for amplification, COR-1 and COR-2, were targeted at the coronavirus polymerase gene designed by GVU: sense 5′ CAC CGT TTC TAC AGG TTA GCT AAC GA 3′and antisense 5′ AAA TGT TTA CGC AGG TAA GCG TAA AA 3′, with expected product size of 311 bp. Five microliters of cDNA was amplified in 45 µL of master mixture containing 5 µL of 10X PCR buffer (Amersham Pharmacia Biotech, Piscataway, NJ), 1 µL of 2 5 mM extra $MgCl_2$, 4 µL of deoxynucleoside triphosphates (dNTPs) (2.5 mM each), 0.5 µL of each primer, 0.3 µL of Taq polymerase (5 U/mL), and 33.7 µL of molecular grade water. One positive control and one negative control were included in each PCR assay. Reactions were performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystem) with the following conditions: at 94°C for 3 min, followed by 45 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and 72°C for 7 min. PCR products were analyzed by gel electrophoresis.

Treatment

All patients received treatment according to a standard protocol. Either a β-lactam plus β-lactamase inhibitor or third-generation cephalosporin in combination with a macrolide or a fluoroquinolone was given to the patient at admission. Per the recommendation of the Hospital Authority of Hong Kong, an antiviral drug (ribavirin 24 mg/kg/day intravenously, together with hydrocortisone 10 mg/kg/day) was administered if the symptoms did not respond within 48 h (The recommendations were found available at: URL: http://www.ha.org.hk/hk/hesd/nsapi/?MIval=ha_view_content&c_id=122711&lang=E. However, the recommendations have since changed and are available at: URL: http://www.ha.org.hk/hesd/nsapi/?MIval=ha_view_content&c_id=123510&hesd_lang=E). Methylprednisolone, in the form of two to three pulsing doses of 500 mg to 1,000 mg a day intravenously, was administered to those with a persistent fever, radiologic evidence progression of lung infiltrates, or signs of respiratory distress despite the initial antiviral-hydrocortisone combination.

Statistical Analysis

Bivariate analysis was performed for epidemiologic, clinical, laboratory, radiologic data, and outcomes by using RT-PCR results as the dependent variable. Data of continuous variables were expressed as mean and standard deviation. Chi-square test was used for categorical variables, and the unpaired Student t test was performed for continuous variables. All significant factors for death with a p value ≤ 0.1 were pooled into a multivariate logistic regression model with backward stepwise analysis to identify the independent predictors for the clinical outcome. A p value ≤ 0.05 (two-tailed) was assumed to be statistically significant. All analyses were performed with the SPSS version 10.0 software (SPSS Inc, Chicago, IL).

Results

Demographic Findings

On admission, nasopharyngeal RT-PCR was performed on 90 male and 128 female patients (mean age 39.6 ± 14 years). All patients, except six, were Chinese; two were Indonesian, and four were Filipino. Twenty-one of the patients (10%) were healthcare workers, including 5 clinicians, 9 nurses, 5 ward assistants, and 2 allied health workers who worked in the SARS wards. Forty-one patients

(19%) reported having recently traveled to SARS-endemic areas in the 2 weeks before admission; the most common areas visited were in the southern part of China. Our cohort consisted of patients (46.8%) from a local housing estate, the Amoy Gardens. Ten patients (4.6%) had one or more coexisting medical problems: diabetes mellitus (3 cases), a history of cerebrovascular disease (4 cases), ischemic heart disease (3 cases), chronic rheumatic heart disease (1 case), hypertrophic obstructive cardiomyopathy (1 case), sick sinus syndrome (1 case), cirrhosis of the liver secondary to chronic hepatitis B (1 case), bronchiectasis (1 case), end-stage renal disease (1 case), Sjögren syndrome (1 case), and nasopharyngeal carcinoma (1 case). The proportions of patients from Amoy Gardens that were RT-PCR positive (48%) and negative (45.7%) were not significantly different ($p = 0.76$). Likewise, the proportions of healthcare workers ($p = 0.28$) or patients with coexisting conditions ($p = 0.83$) did not differ significantly.

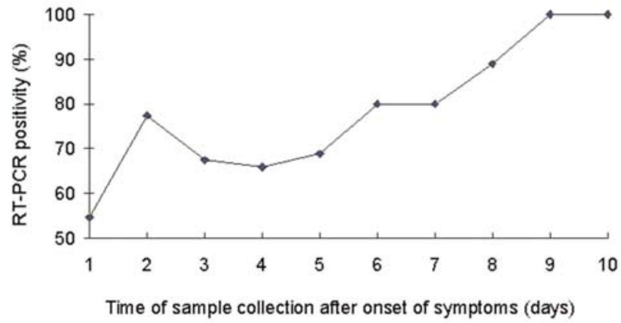

Figure. Percentage of reverse transcription polymerase chain reaction (RT-PCR) positivity at different times of sample collection after onset of symptoms.

RT-PCR Results

NPA samples for RT-PCR were taken from all patients at admission; samples from 156 patients (71.6%) were positive. The mean time from disease onset to sample collection was 4.4 ± 2.3 days. No significant difference in the mean sampling time was found between RT-PCR–positive or –negative patients. The optimal time for sample collection was day 8–10 when 13 of 14 patients (92.9%) were positive (Figure).

Symptoms and Laboratory Findings

The most common clinical features for both RT-PCR–positive and –negative cases included fever, chill, malaise, myalgia, cough, rigor, and headache (Table 1). Shortness of breath and dizziness were significantly higher in RT-PCR–positive patients in bivariate analysis. Vital signs taken on admission (temperature, heart rate, and systolic and diastolic blood pressure) were similar between the two groups. Common laboratory findings included anemia with hemoglobin level <12 g/dL (14.7%), lymphopenia with leukocyte count $<4 \times 10^9$/L (72%), thrombocytopenia with platelet count $<150 \times 10^9$/L (52.3%), hypokalemia with plasma potassium level <3.5 mmol/L (41.3%), hyponatremia with plasma sodium level <135 mmol/L (61.5%), and elevated levels of lactate dehydrogenase >230 U/L (46.6), alanine transaminase > 40 U/L (30.8%), and C-reactive protein (77.8%). By bivariate analysis, lymphopenia and elevated lactate dehydrogenase level on admission were significantly different between RT-PCR–positive and –negative patients (Table 2).

Serologic Test Results

Eighty-seven NPA RT-PCR–positive patients and 33 RT-PCR–negative patients had serologic tests on their paired serum samples 10–14 days apart. Of the positive RT-PCR patients, 74 patients (85.1%) had total antibodies detected by IFA, while serologic tests for 25 patients (75.8%) in the RT-PCR–negative group were positive. Results for 13 patients in the RT-PCR–positive group and 8 patients in the RT-PCR–negative group were negative.

Radiologic Findings

Initial chest radiographs for 210 patients (96.3%) were abnormal. Sixty-five (41.7%) RT-PCR–positive patients and 13 (21%) RT-PCR–negative patients had bilateral chest involvement shown by radiograph. Multifocal radiologic involvement was found in 74 (47.4%) RT-PCR–positive patients and 15 (24.2%) RT-PCR–negative patients. By bivariate analysis, RT-PCR–negative patients were less likely to have abnormal bilateral ($p = 0.01$) and multifocal ($p = 0.003$) radiographs.

Outcomes

The overall 30-day mortality rate was 10.1% (22 patients). Fifty-two (23.9%) patients required intensive-care unit (ICU) admission, and 43 patients (19.7%) needed mechanical ventilation. In nine (4.1%) patients, acute renal failure further complicated SARS. When compared to the RT-PCR–negative patients, the RT-PCR–positive patients were more likely to need treatment in the ICU ($p = 0.002$), require mechanical ventilation ($p = 0.008$), and die ($p = 0.044$) (Table 3).

Predictors of Mortality

Admission parameters, including epidemiologic data, vital signs, and laboratory and chest radiographic findings, were analyzed separately. By bivariate analysis, factors associated with death were age >60 ($p = 0.037$), male sex ($p = 0.007$), major coexisting medical conditions ($p = 0.001$), shortness of breath ($p = 0.005$), total leukocyte count $>4.0 \times 10^9$/L ($p = 0.041$), bilateral chest radiographic involvement ($p = 0.046$), RT-PCR positivity on NPA samples ($p = 0.034$), and pulsing doses of steroid ($p = 0.001$).

Table 1. Symptoms of 218 patients with severe acute respiratory syndrome

Symptoms	Positive RT-PCR for coronavirus n=156 (%)	Negative RT-PCR for coronavirus n=62 (%)	p value
Time from symptoms onset to sample collection (days)	4.5 ± 2.2	4.2 ± 2.4	0.366
Fever	155 (99)	60 (97)	0.139
Chill	120 (77)	51 (82.3)	0.388
Malaise	103 (66)	34 (54.8)	0.123
Myalgia	84 (53.8)	30 (48.4)	0.467
Cough	66 (42.3)	27 (43.5)	0.867
Rigor	65 (41.7)	27 (43.5)	0.800
Headache	49 (31.4)	25 (40.3)	0.210
Anorexia	37 (23.7)	14 (22.6)	0.858
Sputum	28 (18)	13 (21)	0.607
Shortness of breath	36 (23)	5 (8.1)	**0.012**
Dizziness	23 (14.7)	17 (27.4)	**0.029**
Diarrhea	22 (14.1)	9 (14.5)	0.937
Sore throat	21 (13.5)	10 (16.1)	0.611
Runny nose	15 (9.6)	9 (14.5)	0.297
Chest pain	13 (8.3)	8 (12.9)	0.302
Vomiting	12 (7.7)	5 (8)	0.926
Palpitation	2 (1.3)	2 (3.2)	0.320
Hemoptysis	1 (0.6)	1 (1.6)	0.497
Confusion	1 (0.6)	1 (1.6)	0.497

[a]Fisher exact test was applied if p value was <5; RT-PCR, reverse transcription polymerase chain reaction; p values in bold are significant.

By multivariate analysis, independent predictors of 30-day mortality were RT-PCR positivity on NPA samples (odds ratio [OR] 6.4; 95% confidence interval [CI] 1.1 to 38.0; p = 0.038), shortness of breath on admission (OR 3.9; 95% CI 1.2 to 12.3; p = 0.02), presence of important coexisting condition (OR 13.4; 95% CI 3.1 to 58.2; p = 0.001), total leukocyte count >4.0 x 10^9/L (OR, 6.94; 95% CI 1.18 to 41.6; p = 0.033), and pulsing doses of methylprednisolone (OR 26.0; 95% CI 4.4 to 154.8; p = 0.001) (Table 4).

Table 2. Vital signs and laboratory findings in 218 patients with severe acute respiratory syndrome

	Positive RT-PCR for coronavirus (n = 156)	Negative RT-PCR for coronavirus (n = 62)	p value
Vital sign upon admission			
Temperature (°C)	38.5 ± 0.9	38.4 ± 0.9	0.774
Heart rate	95 ± 14	98 ± 16	0.571
Systolic blood pressure	127 ± 18	130 ± 19	0.503
Diastolic blood pressure	71 ± 11	73 ± 12	0.450
Laboratory findings upon admission			
Hemoglobin level (g/dL)	13.3 ± 1.4	13.0 ± 1.6	0.160
Leukocyte count (x 10^9/L)	5.5 ± 2.7	5.5 ± 1.9	0.954
Neutrophil count (x 10^9/L)	4.3 ± 2.6	4.2 ± 2.3	0.885
Lymphocyte count (x 10^9/L)	0.8 ± 0.3	0.9 ± 0.3	0.045
Platelet count (x 10^9/L)	155 ± 55	166 ± 50	0.137
Prothrombin time (sec)	12 ± 2	12 ± 1	0.396
Activated partial thromboplastin time (sec)	35 ± 10	33 ± 5	0.094
Sodium level (mmol/L)	134 ± 4	134 ± 3	0.423
Potassium level (mmol/L)	3.6 ± 0.5	3.5 ± 0.4	0.787
Urea level (mmol/L)	3.7 ± 1.8	3.6 ± 4	0.200[a]
Creatinine level (mmol/L)	74 ± 19	80 ± 79	0.885[a]
Albumin level (g/L)	37± 4	38 ± 5	0.112
Globulin (g/L)	33 ± 5	33 ± 4	0.737
Bilirubin (mmol/L)	9 ± 6	8 ± 5	0.798
Alkaline phosphatase (IU/L)	75 ± 58	67 ± 33	0.245[a]
Alanine aminotransferase (IU/L)	43 ± 41	33 ± 30	0.051[a]
Creatinine phosphokinase (IU/L)	422 ± 1987	189 ± 391	0.118[a]
Lactate dehydrogenase (IU/L)	287 ± 141	208 ± 67	**0.001[a]**

[a]Comparison made after log-transformation of data; RT-PCR, reverse transcription polymerase chain reaction; p values in bold are significant.

Table 3. Clinical progress and outcome on day 30 after admission

Clinical progress/outcome	Positive RT-PCR for coronavirus n=156 (%)	Negative RT-PCR for coronavirus n=62 (%)	p value
Patients requiring ICU care	46 (29.5)	6 (9.7)	0.002
Patients requiring mechanical ventilation	38 (24.4)	5 (8.1)	0.008[a]
Patients developing acute renal failure	8 (5.1)	1 (1.6)	0.451[a]
Death	20 (12.8)	2 (3.2)	0.044[a]

[a]Fisher exact test was applied if number was <5; RT-PCR, reverse transcription polymerase chain reaction.

Discussion

In general, the epidemiologic background, clinical presentation, laboratory findings, and radiologic findings of our patients were similar to those of other reports (3–5). The clinical features of our cohort were rather nonspecific, with fever, chills, malaise, and myalgia being the most common. Radiologic features of our patients were similarly nonspecific. Anemia, lymphopenia, and thrombocytopenia were common on admission. These symptoms might reflect peripheral consumption or bone marrow suppression by the infection. Elevation of alanine transaminase, C-reactive protein, and lactate dehydrogenase levels was frequently observed; this finding might indicate extensive tissue damage.

Currently, the definition of SARS is mainly clinical, and diagnosis is made by exclusion of pneumonia from other known causal agents. However, patients with similar clinical scenarios may not be infected by the same agent, and placing them in the same location may spread infection. Unfortunately isolating every patient is not possible, especially with a large cohort. An early, rapid, and reliable test is needed. After coronavirus was recognized as the putative agent for SARS, diagnostic tests have burgeoned rapidly over the past 2 months. However, serologic tests cannot offer an early diagnosis since, despite their remarkable specificity, they require approximately 3 weeks for the total antibodies to become detectable (2). Electron microscopy and viral culture are not sensitive and convenient enough for general use. Inevitably, clinical characteristics are used solely for the diagnosis of SARS, despite the condition's nonspecific nature.

RT-PCR for coronavirus on NPA samples appears to be the best supportive test for an early and firm diagnosis. However, the sensitivity of this test varies, and standardization of the test has not been unified. The test we used was qualitative and had good sensitivity (71.6%). In our study, the mean time between onset of symptoms and sample collection was 4.3 days. Peiris et al. reported that the sensitivity for RT-PCR was 32% at a mean 3.2 days after onset (3). In our study, the best time for sampling was on days 8 to 10 after onset of symptom. During that period, >90% of the samples were positive. However, since the condition of SARS patients deteriorated both clinically and radiologically during this time, waiting 8–10 days to make a firm diagnosis or to plan for appropriate therapy is not possible (3). The mean time of sample collection did not differ significantly between RT-PCR–positive or –negative patients, suggesting that the difference in outcomes between these two groups could not be explained by the discrepancy in the duration of their symptoms before admission. Whether the difference in primers used in the RT-PCR testing or infection by a another agent, such as metapneumovirus or *Chlamydia* species (6), could also contribute to the discrepancy in sensitivity is not known.

Peiris et al. reported that the sensitivity of stool samples for RT-PCR tests was 97% (mean of 14.2 days) (3). In our study, the overall positive rate was 30%. However, RT-PCR was performed on stool specimens only when diarrhea developed in patients. Inconsistency in the timing of sample collection might also contribute to the low positive rate. In patients with negative RT-PCR results on NPA samples, none had a stool sample positive by RT-PCR. Hence, RT-PCR on stool specimen could not provide an early diagnosis of coronavirus infection in our cohort.

Patients with a positive RT-PCR result on admission had adverse outcomes in term of survival, ICU care, and assisted ventilation, when compared to patients with negative RT-PCR results. Therefore, the patients with overwhelming disease had more viral shedding, which may have been more readily detected. Despite the satisfactory sensitivity that we demonstrated, the test might not provide the information for quantitative analysis. Hence, a negative result might not represent low viral load in patients and vice versa. A quantitative RT-PCR could give us some idea as to the correlation between the viral concentration and disease progression. Peiris et al. reported that the max-

Table 4. Multivariate analysis on risk factors associated with 30-day mortality[a]

Risk factors	Adjusted OR (95% CI)	p value
Significant coexisting conditions	13.4 (3.1 to 58.2)	0.001
Shortness of breath on admission	3.9 (1.2 to 12.3)	0.020
Total leukocyte count >4.0 x10^9/L at admission	6.94 (1.18 to 41.6)	0.033
Positive RT-PCR on NPA	6.4 (1.1 to 38.0)	0.038
Use of pulsing doses of steroid	26.0 (4.4 to 154.8)	0.001

[a]OR, odds ratio; CI, confidence interval; RT-PCR, reverse transcription polymerase chain reaction; NPA, nasopharyngeal aspirates.

imal viral replication by quantitative RT-PCR occurred by approximately day 10, but the clinical worsening seemed to lag behind this peak (3). Although we could not quantify the maximal viral shedding, the maximal RT-PCR positivity did fall on approximately day 10. Peiris et al. also demonstrated that an initial positive RT-PCR result had no correlation to development of an acute respiratory distress syndrome. In our multivariate analysis model, however, initial RT-PCR positivity on NPA was an independent predictor for a worse outcome, rather than a previously reported factor, such as a coexisting condition (3–5). Although quantitative RT-PCR was not performed on samples from our patients, since the test was not available at that time, a qualitative RT-PCR result might alert the clinician to watch out for possible clinical deterioration, especially when the former test was in its infancy for common use. The relationship between viral load on NPA and outcome should be further investigated.

The clinical outcomes of RT-PCR–positive patients are worse in general when compared to RT-PCR–negative patients, and their chest radiographs show more bilateral and multifocal haziness. A higher level of lactate dehydrogenase was observed in the RT-PCR–positive patients, which might indicate more extensive lung tissue injury, as indicated in other SARS patients with poor outcome (4). Whether the lower lymphocyte count in RT-PCR–positive patients suggests more extensive viral infection remains to be clarified. Use of pulsing doses of methylprednisolone could result in clinical improvement and the resolution of radiologic infiltration in some of our patients. However, its immunosuppressive effect could also predispose a patient to secondary nosocomial infection and subsequent death.

How to handle negative results in RT-PCR testing is a problem. In accordance with the World Health Organization's (WHO) recommendations, a negative result has at least two possibilities (7). First, it may indicate a false-negative result caused by low viral load or inappropriate timing of sample collection. Second, another infectious or a noninfectious agent may be the cause of SARS instead of coronavirus. Finally, a negative RT-PCR result on admission may indicate early elimination of the virus by an effective and harmonious immunologic response. Serologic tests are thus important in identifying SARS infections, although the diagnosis could not be made early enough to prompt an appropriate action. In our patients, RT-PCR and serologic results were in concordance. The sensitivity of RT-PCR test was 74.7% when an antibody test was used as a standard, which can be explained by the variation in the technique and timing of sampling. The optimal timing for the RT-PCR test is unknown. The problem of finding an appropriate sampling time was taken in account for the RT-PCR–positive patients with negative serologic results, since they suggested that antibodies could be detected at 21 days (8) instead of 10–14 days, as in our cohort. In addition, the PCR test may be overly sensitive, which may be why WHO has advised clinicians to confirm a positive RT-PCR result by repeating the test with the original sample or testing the sample in a secondary laboratory so as to increase its specificity (8).

Results of both RT-PCR and antibody tests were negative in eight patients; all of these patients had signs and symptoms that were clinically, radiologically, or biochemically well-matched with SARS, and they were given treatment, including ribavirin and steroid. Pulsing doses of steroid was also used in two of these patients. In addition to the sample timing, these patients could represent a milder spectrum of the disease with little antibody stimulation or inconspicuous coronavirus RNA level, or simply infection other than coronavirus. Antibody production may have been suppressed because of steroid administration.

Because RT-PCR testing has not been standardized, the test still varies in sensitivity and specificity, and we are still confronted with a clinical dilemma in terms of infection control and management. Furthermore, the controversy of medication in the management of SARS has never been settled. Current treatment guidelines proposed by the Hospital Authority of Hong Kong are still in use despite the adverse effects of the suggested treatment (The recommendations were found available at: URL: http://www.ha.org.hk/hk/hesd/nsapi/?Mlval=ha_view_content&c_id=122711&lang=E.)Without a reliable and rapid RT-PCR test for diagnosis, patients mislabeled as having SARS will be offered treatment that they do not need.

Our results indicate that a positive nasopharyngeal RT-PCR result on admission, from the current standard, should raise the possibility of SARS in appropriate clinical settings and should alert the clinician of the possible clinical deterioration of the patient. Furthermore, clinicians should consider repeating the qualitative RT-PCR test or performing quantitative RT-PCR test for a previously RT-PCR–negative patient. Drug treatment for this group of patients may be withheld or delayed, especially if effective and reliable treatment has not been found.

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Dr. Tsang is a medical officer currently working in the Infectious Disease Unit of Princess Margaret Hospital. He is responsible for the management of SARS patients during the outbreak of this disease in Hong Kong.

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West Nile Virus Infection in Nonhuman Primate Breeding Colony, Concurrent with Human Epidemic, Southern Louisiana

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During the summer of 2002, an epidemic of West Nile meningoencephalitis occurred in southern Louisiana. Following the outbreak, blood samples were collected from 1,692 captive rhesus monkeys (*Macaca mulatta*), pigtail macaques (*M. nemestrina*), and baboons (*Papio* spp.) that were permanently housed outdoors at a nonhuman primate breeding facility in St. Tammany Parish, Louisiana. The serum samples were examined for antibodies to West Nile virus (WNV). Overall, 36% of the captive nonhuman primates had WNV antibodies; comparison of these samples with banked serum samples from previous blood collections indicated that the animals were infected subclinically from February to August 2002. WNV activity was demonstrated in surveillance at the nonhuman primate-breeding colony and in the neighboring community during this same period. The high infection rate in this captive nonhuman primate population illustrates the intensity of WNV transmission that can occur silently in nature among other susceptible vertebrates during epidemic periods.

Since its first appearance in New York City in 1999, West Nile virus (WNV) has spread rapidly across the North American continent, infecting a wide range of avian and mammalian species (1,2). Within 3 years of its initial appearance, WNV activity was reported in 44 of the 48 states that comprise the continental United States (2). During 2002, a total of 3,389 human cases and ~15,000 equine cases of WNV-associated illness were reported by the Centers for Disease Control and Prevention (CDC) and the U.S. Department of Agriculture (USDA), respectively (2). In the same year, a total of 14,122 WNV-infected dead birds, representing 94 different avian species, were reported (2). Despite the increasing number of reported West Nile cases and the wide vertebrate host range of this emerging flavivirus, surprisingly little information is available on the true prevalence of WNV infection among humans and animals living within newly epidemic regions in North America. This paucity of information is partly due to the method in which WNV infections are recorded: only recognized cases of clinical illness or death are usually reported.

On the basis of retrospective serosurveys conducted in New York City in 1999 and 2000, symptomatic illness develops in approximately 20% of persons infected with WNV and approximately 1 in 150 human infections results in meningoencephalitis, the most commonly reported form of WNV-associated illness (1,3). Serosurveys among equines living in WNV-endemic areas, as well as results of WNV experimental infections of horses, indicate that most equine infections are inapparent or result in only mild clinical illness (4–7). Most reported WNV-associated bird deaths in the United States have been in crows and blue jays (2,8), two species with a relatively high case-fatality rate; however, many other bird species are naturally infected with the virus and have few or no deaths (8–11). Because the current WNV surveillance systems in the United States are largely disease- and death-based (cases of human meningoencephalitis, equine encephalitis, dead birds), the actual prevalence of WNV infection among humans and other susceptible vertebrate hosts and the intensity of virus transmission are underestimated.

During 2002, the state of Louisiana reported 319 human cases of WNV-associated illness; 71% of these cases had WNV meningoencephalitis (2). Most of the West Nile cases were in the southeastern portion of the state. St. Tammany Parish, which is located in that region of the state, was a focus of intense WNV activity in 2002 and

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recorded 40 human cases (12). The Tulane National Primate Research Center (TNPRC) is located in St. Tammany Parish and houses large outdoor breeding colonies of baboons and macaques. The availability of sequential serum samples from these animals during a period of known epidemic WNV activity prompted us to examine some of the animals for serologic evidence of recent WNV infection. The results of these studies are reported here and indicate that approximately 36% of the nonhuman primates tested from the breeding colony were naturally infected with WNV during the 2002 transmission season.

Methods

Study Site

TNPRC is located on 500 acres of land in Covington, St. Tammany Parish, Louisiana, approximately 56 km north of New Orleans. TNPRC has approximately 5,000 nonhuman primates in its research and breeding populations. Approximately 4,000 of these animals are housed in outdoor social groups for breeding. The animal care program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and is registered with the USDA as a class B animal dealer and as a research institution.

The center's breeding colony is situated on approximately 100 acres of land and comprises 223 outdoor holding compounds, which consist of corrals and corncribs. Corrals are constructed of chain link with earthen floors composed of clay and topsoil (Figures 1 and 2). Most corrals have grass coverage. Corncribs are constructed of galvanized metal and are completely covered on top, open with wire on the sides, and have cement flooring. All corrals and corncribs have perching, which is sheltered. The corrals have additional shelter in the form of walled and covered areas, concrete culverts, and calf huts to allow all of the animals to have shelter in inclement weather.

Because the regional climate is mild, animals in the breeding colony are housed outdoors throughout the year. Animals are provided a commercial nonhuman primate diet, fed once a day, and supplemented with fruit 3 days per week. Additionally, foraging feed (cracked corn, seed) is spread throughout the corrals. Water from the Center's artesian well is available ad libitum through an automated Lixit system (Napa, CA).

The breeding colonies housed at the center consist of the TNPRC Conventional Breeding Colony and the TNPRC Specific Pathogen Free Colony, both of which consist of rhesus monkeys (*Macaca mulatta*). TNPRC also houses the breeding colonies for the Washington National Primate Research Center (WaNPRC), which consists of both baboon (*Papio* spp.) colonies and Specific Pathogen Free pigtail macaques (*M. nemestrina*).

Figure 1. Aerial view of the nonhuman primate outdoor holding compounds at the Tulane National Primate Research Center. Approximately 4,000 animals are housed here in social groups for breeding.

Every animal at the TNPRC has a unique identifying number tattooed on the animal's chest or medial thigh. A secondary form of identification is also used in breeding colony animals and consists of neck chains and tags, dye marks, or subcutaneous microchip transponders. Animals are observed for illness twice a day, and ill animals are moved to indoor clinical areas for diagnosis and treatment on a daily basis. All animals in the breeding colony are routinely examined a minimum of twice annually as part of the preventive medicine program.

Sampling Technique

The animals sampled for this study were from the TNPRC SPF colony and the WaNPRC SPF and baboon colonies. At the time of inventory, physical examination, anthelmintic treatment, pregnancy determination, tattooing, and blood sample collection were performed as part of the semiannual preventive medicine program. All inventory procedures were performed in ketamine HCl (10 mg/kg IM) anesthetized animals. Blood was collected from the

Figure 2. Inside view of one of the outdoor holding corrals at the Tulane National Primate Research Center.

femoral vein after preparation of the overlying skin with alcohol. Twenty-two–gauge needles and a Vacutainer collection system were used for blood collection. All procedures performed on animals were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee.

Serologic Studies

After blood collection, serum samples were removed and frozen at –20°C. These samples were subsequently transported on dry ice to the University of Texas Medical Branch, where all serologic testing was done. Initially, all primate sera were screened by hemagglutination-inhibition (HI) test against dengue-1 (DENV-1), DENV-2, yellow fever (YFV), St. Louis encephalitis (SLEV), and West Nile (WNV) antigens. The antigens were prepared from brains of newborn mice injected intracerebrally with each of the flaviviruses; infected brains were treated by the sucrose-acetone extraction method (13,14). Primate serum samples were tested by HI at serial twofold dilutions from 1:20 to 1:5120 at pH 6.6 (WNV and SLEV) or 6.4 (YFV and DENV) with 4 U of antigen and a 1:200 dilution of goose erythrocytes by using established protocols (13,14).

A subsample of the WNV antibody–positive samples by HI test was also examined by complement fixation (CF) and plaque reduction neutralization test (PRNT). CF tests were performed by a microtechnique (13) with two full units of guinea pig complement and antigen titers of ≥1:32. Titers were recorded as the highest serum dilutions giving +3 or +4 fixation of complement on a scale of 0 to +4.

PRNT on the serum samples was performed by a previously described technique (13) in 24-well, Vero microplate cultures, using a fixed virus inoculum (~100 PFU) against varying serum dilutions (1:10 to 1:20,480). For PRNT, the Egypt 101 strain of WNV (13) was used. Virus plaques were read on day 4; ≥90% plaque reduction was used as the endpoint.

Mosquito Surveillance

Because WNV activity had occurred in Louisiana in 2001, an initial survey was conducted on the grounds of the TNPRC from January 29 to April 5, 2002, to determine the potential for WNV transmission to the captive nonhuman primates. Larval mosquito sites were identified by using standard dipping measures to determine larvae and pupae density. At this time, some adult mosquitoes were also collected by using modified dry ice-baited CDC light traps, although assay for WNV was not performed. Results of this survey were used to properly target control strategies to problem areas within the corral facility. Strategies used during this period included larval source reduction (maintenance of ditches and proper drainage) and application of a mosquito growth regulator (Altosid) (Wellmark International, Schaumburg, IL). A subsequent partnership forged with the local mosquito control agency (St. Tammany Mosquito Control) enabled aerial applications of the chemical pesticide Dibrom (AMVAC, Commerce, CA). Throughout the season, seven aerial applications of Dibrom were sprayed at 0.75 oz per acre. A second survey of adult mosquitoes was conducted from July 29 to October 31, 2002.

Two types of traps were used in the mosquito surveillance program. Modified dry ice-baited CDC light traps (Clarke Environmental Mosquito Mgmt., Inc., Roselle, IL) to collect host-seeking mosquitoes were set 1 m from the ground. Gravid traps (John W. Hock, Gainesville, FL) targeting primarily gravid *Culex quinquefasciatus* were placed on the ground in a shaded area and filled with 1 to 2 inches of water and fish emulsion. In this study, one light trap and one gravid trap were placed at four sites around the perimeter of the nonhuman primate breeding corrals and the main campus. Mosquito traps were positioned weekly and operated for 12 hours each collection period. Upon collection, the mosquitoes were immobilized on dry ice and then stored at –20°C. The mosquitoes were later placed on a chill table and identified to species (15).

Results

Serosurvey on Captive Primates

A total of 1,692 primates in the breeding colony at TNPRC were sampled from August to November 2002. The species breakdown of the animals sampled was as follows: 726 rhesus macaques, 563 pigtail macaques, and 403 baboons. All serum samples from these animals were screened by HI test against DENV-1, DENV-2, YFV, SLEV, and WNV antigens; a subset of the WNV antibody positives also were tested by CF and PRNT. The results of serologic tests are summarized in Tables 1–5.

A total of 726 *M. mulatta* were bled between August and November 2002 and tested by HI test for WNV antibodies; 286 (39.39%) of the serum samples were positive

Table 1. Prevalence of West Nile virus hemagglutination inhibition (HI) antibodies by age group among captive rhesus macaques in the breeding colony at the Tulane National Primate Research Center by age group, August–November 2002

Age group (y)	No. tested	No. positive	% positive
0 to <1	8	1	12.50
1 to <2	293	96	32.76
2 to <4	349	141	40.40
4 to <6	61	43	70.49
6 to <8	3	1	33.33
8 to <10	7	3	42.86
10 to <12	2	1	50.00
12 to <14	2	0	0.00
≥14	1	0	0.00
Total	726	286	39.39

Table 2. Results of hemagglutination inhibition (HI) tests on blood samples collected in August 2002 from eight West Nile-seropositive rhesus macaques living outdoors at the Tulane National Primate Research Center[a]

	HI antibody titer				
Animal number (mo bled)	DENV-1	DENV-2	YFV	SLEV	WNV
CK62	1:40[b]	1:20	1:20	1:160	1:320
CL07	1:40	1:20	1:20	1:160	1:320
CL11	1:40	1:40	1:20	1:160	1:640
CL80	1:40	1:40	1:40	1:160	1:640
CL93	1:40	1:40	1:40	1:160	1:640
CM11	1:20	1:20	0	1:20	1:80
CP90	1:40	1:20	1:20	1:40	1:160
CR36	1:40	1:20	1:20	1:80	1:320

[a]DENV, dengue virus; YFV, yellow fever virus; SLEV, St. Louis encephalitis virus; WNV, West Nile virus.
[b]Highest positive serum dilution

as shown in Table 1. Of the total, 462 were female, and 264 were male; the prevalence of WNV antibodies by sex was 42.2% and 34.5%, respectively. Table 1 summarizes the WNV antibody rates by age group. The lowest WNV antibody rate was in the 0 to <1 age group and the highest was in the 4 to <6 age group.

All serum samples were tested in HI tests against DENV-1, DENV-2, YFV, SLEV, and WNV antigens. Although cross-reactions were observed with these other flavivirus antigens, in all cases the HI antibody titers were highest with WNV antigen. Table 2 illustrates the results obtained with eight HI-positive rhesus serum samples collected in August 2002. The pattern of the HI antibody response in these animals was characteristic of a single flavivirus infection with WNV (16,17).

A subsample of 40 WNV antibody-positive rhesus sera from August were also examined by CF and PRNT to confirm the HI results. The CF and PRNT results on the same eight HI positive serum samples are summarized in Table 3. Results of the latter tests confirmed that the nonhuman primates had been infected with WNV.

To determine more precisely when the animals had been infected with WNV, serum samples collected 6 months earlier (February or March 2002) from 40 of the antibody-positive rhesus macaques were recovered from the serum bank and examined by HI test together with serum samples collected from the same animals in August or September. All of the samples from February to March 2002 were WNV antibody-negative, whereas, serum samples from the same animals from August or September 2002 were WNV antibody-positive. These data indicate that the primates were infected from February to September 2002.

We compared the prevalence of WNV antibodies in 712 captive rhesus macaques by their cage location within the 100-acre breeding colony. The resulting data (not shown) indicated that the risk for infection was similar regardless of cage location.

Table 4 summarizes HI test results on 563 pigtail macaques (*M. nemestrina*) from the TNPRC breeding colony by age group. Overall, 20.25% of these animals had WNV antibodies. The antibody rates among the various age groups were similar. The antibody prevalence by cage location was compared; and as observed with the rhesus macaques, the antibody rates among cage groups were not markedly different.

Table 5 shows the prevalence of WNV antibodies by age group among 403 captive baboons (*Papio* spp.) bled from August to November 2002 at the TNPRC. Overall, the prevalence of WNV antibodies among the baboons was higher (51.36%) compared to the rhesus (39.39%) and pigtail (20.25%) macaques. Except for the 0 to <1 group, the

Table 3. Results of complement-fixation (CF) and plaque reduction neutralization (PRNT) tests done on sera of eight WNV-seropositive rhesus macaques bled in August 2002 at the Tulane National Primate Research Center[a]

	CF antibody titer					
	Viral antigen					PRNT antibody titer with WNV
Animal no.	DENV-1	DENV-2	YFV	SLEV	WNV	
CK62	0	0	0	16/8[b]	128/≥32	1:320[c]
CL07	0	0	0	8/8	64/≥32	1:160
CL11	0	0	0	8/8	64/≥32	1:1280
CL80	0	0	0	16/8	128/≥32	1:80
CL93	0	0	0	32/8	128/≥32	1:320
CM11	0	0	0	0	16/≥32	1:160
CP90	0	0	0	0	32/≥32	1:160
CR36	0	0	0	8/8	64/≥32	1:320

[a]Viral antigens used in CF test: DENV, dengue virus; YFV, yellow fever virus; SLEV, St. Louis encephalitis virus; WNV, West Nile virus.
[b]CF titers expressed as reciprocal of highest antibody titer/reciprocal of antigen titer.
[c]PRN titers expressed as highest serum dilution producing ≥90% plaque reduction.

Table 4. Prevalence of West Nile virus hemagglutination inhibition (HI) antibodies among captive pigtail macaques at the Tulane National Primate Research Center by age group, August–November 2003

Age group (y)	No. tested	No. positive	% positive
0 to <1	49	5	10.20
1 to <2	72	12	16.67
2 to <4	126	25	19.84
4 to <6	94	24	25.53
6 to <8	78	22	28.21
8 to <10	93	14	15.05
10 to <12	33	6	18.18
12 to <14	6	2	33.33
≥14	12	4	33.33
Total	563	114	20.25

WNV antibody rates among the various age groups were similar. The baboons are housed in three contiguous large breeding enclosures. The higher density of animals in the baboon cage may make them more attractive to host-seeking mosquitoes and might account for the higher infection rate among this species.

Mosquito Surveillance Studies

A second survey of adult mosquitoes was made in the breeding colony at TNPRC from July 29 to October 31, 2002, to determine the vector abundance and species composition, after the intensive mosquito control effort in St. Tammany Parish in response to the human WNV epidemic. Mosquito collections were made 1 night each week. A total of 718 adult mosquitoes were collected during this period; the species composition is shown in Table 6. Virus isolation was attempted by culture in Vero cells, but all mosquito pools yielded negative results. Mosquitoes of the genus *Culex* comprised 40.9% of the total collected. Because of Tropical Storm Isidore and Hurricane Lili, which inundated south Louisiana with rain in September and October 2002, relatively large populations of flood water mosquitoes (i.e. *Aedes vexans, Ochlerotatus infirmatus, Psorophora ferox*) were present in collections made during those months.

Discussion

The results of our serosurvey indicate that approximately 36% of the primates housed outdoors at TNPRC were infected with WNV during the spring or summer of 2002. This timing coincides with the observed pattern of WNV activity documented in the Covington area during surveillance by the St. Tammany Mosquito Control, Louisiana State Department of Health, and CDC (12). The presence of WNV in 2002 was first detected in the Covington area with sentinel chicken seroconversions on June 6 and June 10. After these events, a pool of *Cx. quinquefasciatus* from a gravid trap at the TNPRC breeding corrals tested positive for the virus on June 11 (testing done at Louisiana State University, Baton Rouge). The following week (June 13–21), a dead crow, three horses, and five blue jays in Covington also tested positive for WNV. Viral testing of birds was suspended in the parish after 8 WNV-infected dead birds were reported.

Despite the number of nonhuman primates infected with WNV during the summer of 2002, no compatible clinical illness or neurologic disease was observed in any of the animals. In addition, no changes were noted in monthly data on illness and death derived from the breeding colony during the time of seroconversion or thereafter. Animals at TNPRC are observed twice a day for illness, and ill animals are moved indoors to a clinical area for diagnosis and treatment. Although antibodies to WNV have been reported before in wild nonhuman primates (18), relatively little is known about the pathogenesis of natural WNV infection in nonhuman primates. The available data (19–23) suggest that intracerebral or intranasal inoculation of rhesus and cynomolgus (*Macaca fasciacularis*) monkeys may result in encephalitis and death, whereas intravenous or subcutaneous inoculation of these monkeys generally results in an asymptomatic infection or mild febrile illness.

In view of the frequency of WNV infection observed among nonhuman primates in the breeding colonies at TNPRC, one obvious question is whether these animals could serve as amplifying hosts of the virus to infect biting mosquitoes. We recently completed an experiment in which 5 seronegative rhesus monkeys were inoculated subcutaneously with a 1999 New York strain of WNV. The animals were bled daily to determine the level and duration of viremia. Clinical signs of illness did not develop in any animal, and the viremia was of short duration (1–5 days) and did not exceed $10^{2.0}$ infectious units per mL (23). This level of viremia is much lower than that reported for many avian species (11) and is below the titer considered infectious for *Cx. quinquefasciatus* (11,24), the presumed major vector of WNV in Louisiana.

Table 5. Prevalence of West Nile virus hemagglutination inhibition (HI) antibodies among captive baboons at the Tulane National Primate Research Center by age group, August–November 2002

Age group (y)	No. tested	No. positive	% positive
0 to <1	57	14	24.56
1 to <2	60	23	38.33
2 to <4	76	39	51.32
4 to <6	47	33	70.21
6 to <8	36	27	75.00
8 to <10	25	17	68.00
10 to <12	39	61	41.03
12 to <14	29	19	65.52
≥14	34	19	55.88
Total	403	207	51.36

The results of our serosurvey illustrate the intensity of WNV transmission that can occur during periods of epidemic activity. To date, most of the studies of WNV ecology in North America have focused on humans, equines, and a few susceptible bird species. However, WNV, like its close relative Japanese encephalitis virus (25), has an extremely wide host range among vertebrates (2,26–28). The role of some of these other species in the amplification and maintenance of WNV in North America remains to be determined.

The overall prevalence of WNV antibodies (36%) among the 1,692 primates sampled at TNPRC is comparable to WNV infection rates reported in bird populations elsewhere in the United States after human epidemics of West Nile encephalitis. In a retrospective serosurvey in New York City after the 1999 outbreak, Komar et al. (9) reported that 33% of 430 birds sampled had WNV antibodies. Likewise, a retrospective serosurvey at the Bronx Zoo (the epicenter of the 1999 outbreak) found that 34% of captive and wild birds had antibodies (27). During 2001, the Florida Department of Health recorded annual seroconversion rates as high as 54% and 57% among sentinel chickens in some counties where West Nile human and equine cases occurred (8). In a retrospective survey of wild birds in the Houston metropolitan area in the fall and winter of 2002 (WNV was first detected in Texas in the summer of 2002), 34% of 218 bird sera tested had WNV antibodies (RS Tesh and A.P.A. Travassos da Rosa, unpub. data). These infection rates have occurred in affected regions after WNV was introduced. Whether this high level of virus transmission will continue to occur annually remains to be determined.

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Human Hantavirus Infections, Sweden

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The prevalent human hantavirus disease in Sweden is nephropathia epidemica, which is caused by *Puumala virus* and shed by infected bank voles (*Clethrionomys glareolus*). To evaluate temporal and spatial patterns of this disease, we studied 2,468 reported cases from a highly disease-endemic region in northern Sweden. We found that, in particular, middle-aged men living in rural dwellings near coastal areas were overrepresented. The case-patients were most often infected in late autumn, when engaged in activities near or within manmade rodent refuges. Of 862 case-patients confident about the site of virus exposure, 50% were concentrated within 5% of the study area. The incidence of nephropathia epidemica was significantly correlated with bank vole numbers within monitored rodent populations in part of the region. Understanding this relationship may help forestall future human hantavirus outbreaks.

Members of the genus *Hantavirus* (family *Bunyaviridae*) are commonly transmitted to humans through infected rodent excretions and may cause two severe human diseases: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (1,2). Ecologic factors that mediate hantavirus distribution and maintenance in rodent host populations are not well known, but local variations of hantavirus antibody prevalence within host populations have been observed (3). Approximately 60,000–150,000 humans are hospitalized because of hantavirus infections worldwide each year, and to date, no specific treatment is available (4). Nephropathia epidemica, the less severe form of hemorrhagic fever with renal syndrome, is found throughout Europe, especially in Fennoscandia and European Russia (5). Nephropathia epidemica is caused by *Puumala virus* (PUUV), carried naturally and shed by the bank vole (*Clethrionomys glareolus*) (1).

PUUV is the only hantavirus isolated so far in Sweden. Approximately 90% of all nephropathia epidemica cases in this country are found in the four northernmost counties, hereafter denoted as the northern region (6–8). In this area, nephropathia epidemica is, second to influenza, the most prevailing serious febrile viral infection. Incidence rates are, on average, 20 per 100,000 inhabitants per year (6,7). In a randomized and stratified study within northern Sweden, the prevalence of PUUV-specific immunoglobulin (Ig) M antibodies was 5.4% in adult humans, implying that approximately one eighth of human PUUV infections were diagnosed and reported (8). During 1998, a record number of nephropathia epidemica cases were reported in Sweden; 562 were serologically confirmed (9). These observations suggest that in 1998 as many as 4,500 persons in the northern region may have been exposed to and infected by PUUV.

In this study, we evaluated the demographic patterns and activities in humans that are associated with the likelihood of being diagnosed with nephropathia epidemica in the northern region; examined temporal differences in the incidence of this infection to determine seasonal or periodic patterns; determined how nephropathia epidemica at the local level related to bank vole abundance in a restricted part of the region; and examined distribution of this disease within the region.

Materials and Methods

From January 1991 to December 1998, numbers, dates of diagnosis, and demographic details of nephropathia epidemica cases within the northern region (i.e., the counties of Norrbotten, Västerbotten, Jämtland, and Västernorrland) were obtained from laboratory reports from the Departments of Clinical Virology and Clinical Microbiology in Umeå and Boden, Sweden, respectively. Diagnosis was confirmed by the detection of IgM to PUUV with either an immunofluorescence assay or an enzyme-linked immunosorbent assay (10). For the remaining part of the period studied, January 1999–December 2001, numbers and dates of confirmed cases, without demographic details, were obtained from county medical

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officers. The distribution of cases by county, sex, and age groups was compared to the corresponding demographic data for the entire population of the region during the study period (11).

To characterize exposure sites and possible risk behaviors, a questionnaire was sent to 1991–1998 nephropathia epidemica case-patients concerning activity and location when they were likely exposed to PUUV. If no answer was received within 2 months, a second letter was sent; those persons who still did not respond were contacted by telephone. This complementary telephone survey was restricted to patients whose condition was diagnosed during 1997 and 1998. To determine possible biases concerning sex and age classes in the retrieved answers, compared to data regarding the total human populations on those areas, we used G tests (log-linear likelihood models) for analyses of relative frequencies of received questionnaires (12).

The incubation period of nephropathia epidemica ranges from 2 to 5 weeks after infection (13). Because of uncertainty among many patients concerning the date of infection, we pooled cases by the season of diagnosis: winter (January–March), spring (April–June), summer (July–September), and autumn (October–December). We used analysis of variance (ANOVA) to test for variation of nephropathia epidemica incidence within and between counties, years, and seasons. Where significant F-values were observed, we tested for differences within model effects by the Tukey honest significant difference test (12).

The bank vole–trapping data used in the analyses are publicly accessible and originate from a long-term rodent monitoring project in the county of Västerbotten (available from: URL: http://www.eg.umu.se/personal/hornfeldt_birger/bh/sidor/index2.html). The dataset consists of trapping indices obtained from biannual samplings of small mammals. Autumn bank vole abundance was estimated by snap trapping, killing and removing, in late September within a 100 x 100-km area in which 16 trapping areas were regularly distributed, according to the Swedish National Grid. In each of the 16 5 x 5-km subareas, four 1-ha plots were subject to trapping, unless they were on untrappable sites. In all, 58 of 64 possible 1-ha plots were subject to trapping. Habitats mainly comprised managed conifer forests, i.e., *Pinus sylvestris* and *Picea abies* (78%), including clearcut areas, plantation, peat bogs (18%), and agricultural land (4%) equivalent to and reflecting the general environmental composition within the main study area.

The trapping effort per sampling event was normally 150 trap-nights per 1-ha plot. Five traps were set within a 1-m radius (e.g., in runways, at guidelines, or in holes) of each of 10 permanent stations, centered and spaced 10 m apart along the diagonal of the square 1-ha plot, for three consecutive nights. Traps were checked once each morning. The average total trapping effort per sampling occasion was ≈ 8.440 trap nights. The traps were baited with unfried Polish wicks, i.e., wicks dipped in cooking oil and rolled in wheat flour (14), together with pieces of dried apples.

Trapping indices represent the number of voles captured per 100 trapping nights, a reflection of the relative population size on each sampling occasion. The methods and field procedures for this small mammal sampling have been described (15,16). Bank vole–trapping indices from autumn samplings, in the end of September and early October, were used as a predictor of nephropathia epidemica incidence. Human incidence data were transformed by log-normal transformation [incidence' = log (incidence + 1)] and bank vole autumn trapping indices by arcsine transformation [trapping index' = arcsine {(trapping index + 0.5)√0.5}] (12).

Only patients who declared that they were confident about the occasion and site of their exposure were included in the individual-based spatial analysis. Each such site was assigned to the nearest 10 x 10-km node within the Swedish National Grid system, using the geographic information system ArcView (ESRI, Redlands, CA). Density estimations of the distribution of these case-patients within the northern region were then calculated by fixed kernels of 95%, 75%, and 50% probabilities (17). The kernel method is a nonparametric statistical tool to recognize spatial patterns and areas of defined probability and estimated range for the distribution of study objects, e.g. nephropathia epidemica cases, and was most explicitly pictured by Worton (18) as the following: "A scaled-down probability density function, namely the kernel, is placed above each data point and the estimator is constructed by adding the *n* components. Thus, where there is a concentration of points the kernel estimate has a higher density than where there are few points. Because each kernel is a density the resulting estimate is a true probability density function itself."

Results

From 1991 to 1998, a total of 1,724 persons were identified with serologically verified hantavirus infection (1,075 males, 649 females). We received answers to the questionnaires and telephone surveys from 1,305 persons (76%). Of these, 862 were confident about the time and location of hantavirus exposure, and information from them was used for more reliable exposure site identification. There was no bias in the received answers as compared to available human census data regarding county ($G = 4.33$, df = 3, $p = 0.22$), age group ($G = 10.35$, df = 13, $p = 0.67$, age groups <15 years and >79 years were respectively pooled) or sex ($G = 0.78$, df = 1, $p = 0.38$). Nephropathia epidemica occurred in persons of all age groups (range 3–92 years, mean age 47.4 years), but the age distribution of patients was significantly different from the mean age distribution of the entire human population in the region during the study period (G = 813.00, df = 1, $p < 0.001$, Figure 1). Men aged 25 to 74 years and women aged 45 to 59 years were overrepresented among the case-patients, and persons of both sexes <25 years of age were underrepresented (Figure 1). Male patients were over-represented compared to female patients (G = 103.0, df = 1, $p < 0.001$). Of the case-patients who answered the questionnaire and were confident about the exposure event, 82% claimed that it had occurred in or adjacent to a dwelling (year-round residence [54%] or a holiday house [28%]) and that they were engaged in handling firewood (27%), cleaning or redecorating the residence (19%), gardening or handling hay (18%).

When incidence data from 1999 to 2001 were added, a total of 2,468 serologically verified nephropathia epidemica cases were identified in the northern region from January 1, 1991, to December 31, 2001, giving a yearly average incidence of 25 cases per 100,000 inhabitants during the study period (Table, Figure 2). ANOVA showed that the incidence of nephropathia epidemica was not uniformly distributed between counties (F-value = 25.9154, df = 3, $p < 0.001$), years (F-value = 8.3595, df = 10, $p < 0.001$) or seasons (F-value = 19.0528, df = 3, $p < 0.001$), but without interaction of counties and years (F-value = 0.8801, df = 30, p = 0.6474). Tukey tests showed that, on the county level, the inland county Jämtland (Table, Figure 2) had a significantly lower incidence than Västerbotten and Norrbotten (Tukey $p < 0.05$), and Västernorrland had significantly lower incidence than Västerbotten (Tukey $p < 0.05$). Of the 11 years included in the study, 1995, 1998, 1999, and 2001 had a higher incidence of infection than other years (Tukey $p < 0.05$). Of the four seasons, autumn and winter had significantly higher incidence than spring (Tukey $p < 0.05$), but autumn also differed from summer (Tukey $p < 0.05$). The seasonal periods that had significantly highest incidence, i.e., autumn and winter, were further evaluated in relation to the Västerbotten bank vole–trapping indices. We compared the preceding bank vole–sampling results of the current autumn to nephropathia epidemica incidence in autumn and winter. The highest coefficient of determination of nephropathia epidemica incidence from the bank vole index was found during autumn in Västerbotten [Västerbotten incidence'= –0.1907 + 0.8538 (trapping index'), R^2 = 0.7526, $p < 0.001$], followed by Jämtland [Jämtland incidence'= –0.4046 + 0.8348 (trapping index'), R^2 = 0.6004, p = 0.0051]; Norrbotten [Norrbotten incidence'= –0.3632 + 0.9372 (trapping index'), R^2 = 0.5049, p = 0.0143], and Västernorrland [Västernorrland incidence'= –0.4489 + 0.9372 (trapping index'), R^2 = 0.5049, p = 0.0143] (Figure 3). The winter period had high incidence, but the coefficients of determination between bank vole indices and incidences of nephropathia epidemica were very low and statistically significant only for Västerbotten [Västerbotten incidence'= -0.0230 + 0.6998 (trapping index'), R^2 = 0.3753, p = 0.0451], whereas the rest were nonsignificant; Jämtland [Jämtland incidence'= 0.1733 + 0.2216 (trapping index'), R^2 = 0.0627, p = 0.4576]; Norrbotten [Norrbotten incidence'= –0.2749 + 0.8698 (trapping index'), R^2 = 0.3052, p = 0.0780] and Västernorrland [Västernorrland incidence'= –0.0209 + 0.5392(trapping index'), R^2 = 0.1266, p = 0.2829] (Figure 4).

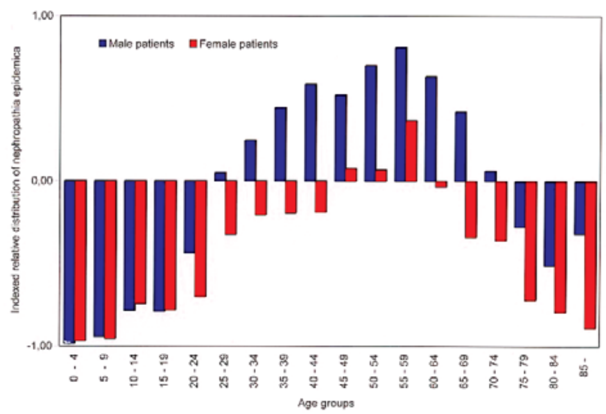

Figure 1. Indexed relative distribution of nephropathia epidemica (NE) cases during 1991 to 1998, within age group and sex (i) in relation to the total human population (pop) in the northern region (NR), where deviations from zero indicate overrepresentation vs. underrepresentation, as calculated by [index]i = $[\{(\#NE)_i|(\#NE)_{NR}\}|\{(\#pop)_i|(\#pop)_{NR}\}–1]$.

On the fine-tuned spatial distribution, based on the 862 confident nephropathia epidemica patients, the Kernel density estimates were strongly skewed towards the coastal areas of the region (Figure 5). Ninety-five percent of the cases were found within 55% of the northern region (158,209 km^2), the 75% Kernel covers 15% of the study area (42,762 km^2); 50% of cases were concentrated within 5% (14,329 km^2) of the study area.

Discussion

We found that the persons at highest risk of having clinical nephropathia epidemica were middle-aged men, a result also observed in other European studies (19–21). However, an earlier study on a stratified and randomly selected number of people from Västerbotten and Norrbotten showed no differences between sexes in the actual prevalence of PUUV infection, and the highest prevalence of IgM antibodies was observed in persons ≥55 years of age (8). Our finding that middle-aged men were overrepresented in relation to sex and age groups, respectively, may reflect age- or sex-related difference in, for

Table 1. Quarterly incidence of nephropathia epidemica per year and county, and autumn trapping indices of bank voles

Counties and Populations	Season	Incidence of notified Nephropathia epidemica cases										
		1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Norrbotten	Jan-March	10.20	7.89	14.60	0.75	6.01	9.08	2.67	15.74	23.25	1.17	11.38
Inhabitants: 263.891	April-June	5.66	5.26	0.00	0.75	1.13	4.92	3.81	18.43	5.42	3.12	1.57
Inhabitants/km^2: 3[c]	July-Sept	1.51	4.51	4.12	0.75	3.01	8.32	1.52	13.44	1.16	1.17	7.46
	Oct-Dec	10.57	12.03	0.75	4.11	8.27	7.57	9.15	19.20	2.32	10.93	12.95
Västerbotten	Jan-March	12.21	8.98	12.01	2.69	7.68	5.00	4.63	10.09	35.84	4.30	9.81
Inhabitants: 257.860	April-June	1.97	1.95	3.10	1.92	3.46	3.46	3.86	13.96	13.24	3.52	8.63
Inhabitants/km^2: 5	July-Sept	8.27	8.98	1.94	3.85	12.29	9.62	3.86	18.23	3.51	3.91	5.49
	Oct-Dec	11.03	7.03	3.10	8.47	10.37	3.85	8.49	30.26	6.23	9.39	10.60
Västernorrland	Jan-March	4.98	3.07	6.91	1.15	17.04	2.34	2.36	7.54	16.04	0.41	8.98
Inhabitants: 256.777	April-June	1.53	0.77	1.54	2.69	2.71	0.78	1.57	7.94	8.02	0.00	5.71
Inhabitants/km^2: 11	July-Sept	0.77	2.68	0.38	4.61	4.26	1.95	1.18	16.28	2.81	2.03	9.38
	Oct-Dec	4.21	4.60	1.92	8.07	4.65	3.12	4.32	30.57	1.60	10.53	17.55
Jämtland	Jan-March	1.47	3.68	3.67	2.20	1.48	3.72	0.75	2.28	3.06	0.00	2.33
Inhabitants: 134.324	April-June	0.74	0.74	0.73	0.00	0.00	0.74	0.75	0.76	2.30	0.00	0.78
Inhabitants/km^2: 3	July-Sept	0.00	2.21	0.73	1.47	2.95	0.00	0.00	8.35	0.77	0.77	3.89
	Oct-Dec	8.09	2.21	1.47	5.87	5.16	2.23	5.26	14.42	1.53	5.40	7.00
No. Bank voles / 100 nights[d]	Sept	4.19	1.94	0.55	2.61	5.09	2.51	1.75	8.39	2.61	2.82	3.49

[a] No. of reported nephropathia epidemica cases per 100.000 inhabitants.
[b] No. of bank voles trapped per 100 trapnights.
[c] Average population density 1991-2001 from census.
[d] Bank vole trapping indices from Västerbotten.

example, risk behaviors, likelihood of seeking medical attention, symptoms, or a combination of these that merit further studies.

The years of the highest incidence of nephropathia epidemica show a periodic pattern similar to that of small mammal population dynamics of northern Sweden, with approximately 3-year intervals between peaks (15,16,22). During these, and most other, years the incidence of this disease peaked in the autumn, with winters also showing a high incidence. However, PUUV transmission among the sexually mature bank voles, within the studied system (23) and in a similar endemic region (24), had been shown to be highest during their reproductive summer period. Within the studied populations, few newly recruited young and immature bank voles had PUUV antibodies (23). The temporal discrepancy on PUUV transmission between conspecific voles vs. voles to humans is likely due to bank voles' abandoning their territoriality in autumn (25), with subsequent extensive individual movements and risk of human dwellings being entered and infested by infected rodents when the climate becomes more harsh (26,27). That rodents invade dwellings is also an observation made from the system of deer mouse (*Peromyscus maniculatus*) and Sin Nombre virus (28). This increased risk for human exposure to PUUV in autumn due to infestation of dwellings by bank voles is consistent with the fact that the within-year vole population usually peaks in autumn (16), when the PUUV-infected bank voles generally appear in highest numbers (23,29). The significant linear correlations of the incidence of nephropathia epidemica within the four counties to the bank vole–trapping indices from Västerbotten demonstrate a spatiotemporal synchronization for the region in bank vole dynamics. Although the strongest association was found in Västerbotten, the pattern was similar for the other counties, albeit with a lower coefficient of determination. Nevertheless, vole abundance in the autumn is a good predictor on the risk of nephropathia epidemica outbreaks during the subsequent autumn and winter. This observation is valuable since a large proportion of cases were identified adjacent to the sampling area of the long-term small mammal study. The ongoing long-term small mammal studies in the Four Corners region of the southwestern United States rapidly detected changes in population densities of the monitored rodent species, several of which were hantavirus hosts

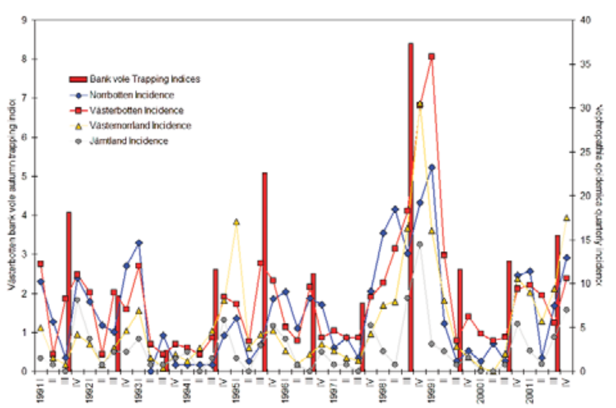

Figure 2. Quarterly incidence of reported nephropathia epidemica cases within respective counties of the northern region from 1991 to 2001, as represented by lines; bars represent annual autumn bank vole–trapping indices.

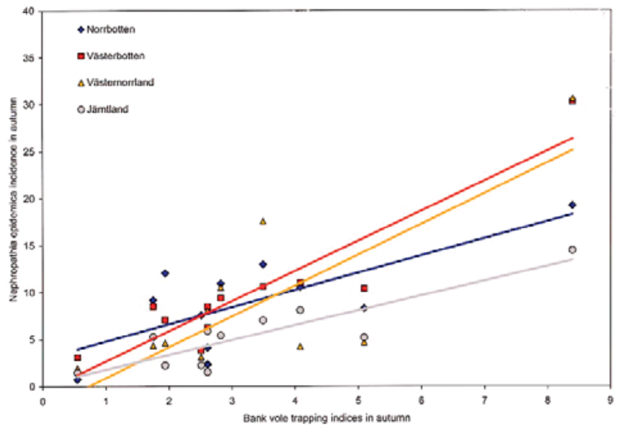

Figure 3. Correlation between incidence on reported nephropathia epidemica cases in respective counties within the northern region in autumn (October–December) and autumn (late September) bank vole–trapping indices in Västerbotten during 1991 to 2001 (data presented nontransformed, before statistical analyses).

(30). Findings like these highlight the relevance of long-term surveys of natural hosts of significant zoonoses. A study during 1985 to 1991 in Västerbotten indicated a highly significant relationship between bank vole–trapping indices and nephropathia epidemica incidence during July through December (31). But the period January–June also showed a highly significant correlation between the number of bank voles in the previous autumn and the incidence of this disease (32). The same relationship, concerning the January–March period in Västerbotten, was significant in our study. However, dynamics of the local bank vole populations have changed in the sense that annual winter declines have become more precipitous (B. Hörnfeldt, pers. comm.).

We are not certain what causes the overall lower incidences in the counties of Västernorrland and Jämtland, compared to the two northernmost counties, Norrbotten and Västerbotten. A likely explanation may be a regional difference in absolute numbers of bank voles, though it is the most common small mammal species throughout the region. That the people who became infected and answered the questionnaire may or may not constitute a random sample of the population available in the respective counties with regard to behavior outside urban settlements is another factor to be considered. Any such differences may cause disparities in frequency of contacts between humans and bank voles and the subsequent risks in acquiring a PUUV infection.

Administrative limitations at the county level are inadequate to describe the actual occurrence of nephropathia epidemica in northern Sweden with any accuracy. An accurate estimate could be made by using the patterns of geographic distribution of the 862 cases (Figure 5), as described herein. Most cases in patients confident about the site of exposure were distributed along the coastal areas of the Gulf of Bothnia, where 50% of the cases clustered within 5% of the study area. This finding coincides with the actual distribution of humans in the region, and so may best explain the spatial occurrence of nephropathia epidemica. However, while the major cities and towns are located within this area, the cases are not associated with urban settlements (8,32). The remaining residents here, in rural areas, represent $\leq$30% of the total population in the northern region (11). The occurrence of nephropathia epidemica in relation to the actual distribution of humans within the region needs to be further evaluated to establish whether the observed pattern is merely a reflection of human population density per se, or if environmental factors also favor and promote hantavirus circulation within local bank vole populations and encounters with humans. Lack of sheltering snow cover should force bank voles to use manmade vole refuges to avoid the harsh climate and predators (33). Thus, the more maritime climate along the coast, in which the period of adequate snow cover is delayed and shortened in comparison to the more continental inland climate, may be a contributing factor to the regional differences in incidence (34).

In reply to our inquiry, persons often stated that sites of human PUUV exposure were woodsheds and woodpiles, where many may have become infected while handling firewood, which stirred up PUUV-contaminated dust particles. These peridomestic bank vole harborages provide a refuge against most predators and shelter from flooding during times of heavy rainfall. Korpela and Lähdevirta (27) observed a correlation between nephropathia epidemica and small rodent occurrence inside rural dwellings, particularly in cupboards, where rodent excretions were deposited. In the present study, cleaning and redecorating

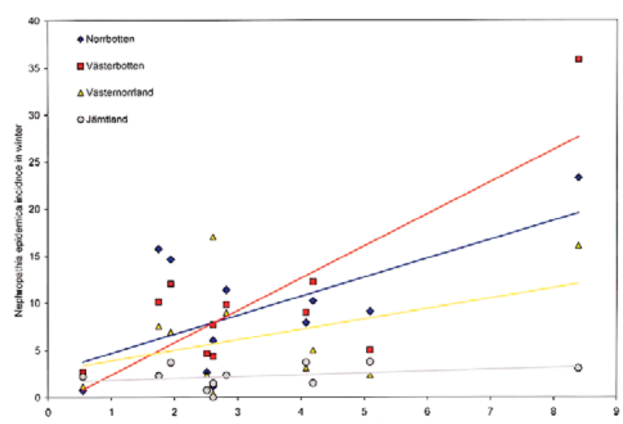

Figure 4. Correlation between incidence on reported nephropathia epidemica cases in respective counties within the northern region in winter (January–March) and previous autumn (late September) bank vole–trapping indices in Västerbotten during 1991 to 2001 (data presented nontransformed, before statistical analyses).

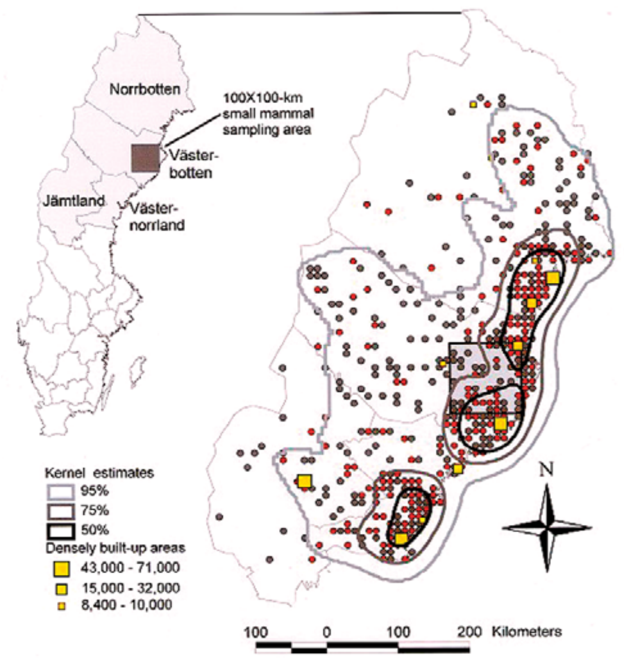

Figure 5. Spatial distribution of nephropathia epidemica infections during 1991 to 1998 in the northern half of Sweden as suggested by patients confident about their site of virus exposure (n = 862). Sites of virus exposure are represented by red (1998 outbreak) and gray dots (remaining years). Densely built-up areas are represented by yellow squares (8,400 to 71,000 inhabitants). Kernel estimates (bold contours) on spatial clustering of cases represent 95%, 75%, and 50% chances of encountering a case among the 862 samples.

were among the activities reported when exposure to PUUV was thought to have occurred. These activities also put persons at risk of inhaling PUUV-contaminated dust particles.

In conclusion, middle-aged persons engaged in activities in or near manmade vole refuges were overrepresented among patients diagnosed with nephropathia epidemica. Most cases were diagnosed during autumn; this finding was more pronounced after an eruption in bank vole numbers the preceding summer and autumn. The spatial distribution of asserted sites of exposure to PUUV was skewed towards the coastal areas of the region. Bank vole dynamics and behaviors, with larger scale movements in autumn and subsequent invasion of human dwellings, together with dense human population in these areas, are among the likely candidates for the observed temporal and spatial patterns. The high human incidence and well-studied rodent community in the present system make it feasible for use as a model system to evaluate environmental factors that may influence PUUV circulation, persistence, and transmission to humans.

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Fatal Spotted Fever Rickettsiosis, Minas Gerais, Brazil

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The emergence and reemergence of a serious infectious disease are often associated with a high case-fatality rate because of misdiagnosis and inappropriate or delayed treatment. The current reemergence of spotted fever rickettsiosis caused by *Rickettsia rickettsii* in Brazil has resulted in a high proportion of fatal cases. We describe two familial clusters of Brazilian spotted fever in the state of Minas Gerais, involving six children 9 months to 15 years of age; five died. Immunohistochemical investigation of tissues obtained at necropsy of a child in each location, Novo Cruzeiro and Coronel Fabriciano municipalities, established the diagnosis by demonstration of disseminated endothelial infection with spotted fever group rickettsiae. The diagnosis in the two fatal cases from Coronel Fabriciano and the surviving patient from Novo Cruzeiro was further supported by immunofluorescence serologic tests.

Infection with *Rickettsia rickettsii,* known as Brazilian spotted fever (BSF) or Rocky Mountain spotted fever (RMSF), occurs in the United States, Canada, Mexico, Costa Rica, Panama, Colombia, Brazil, and Argentina (1–3). Investigations of RMSF often uncover several fatalities (4). A high case-fatality rate is associated with the emergence or reemergence of RMSF after a decade or more of low incidence. Subsequently, the public health concern and educational efforts usually lead to more effective diagnosis, antirickettsial treatment, and a lower case-fatality rate. BSF cases and outbreaks were described in Minas Gerais state, Brazil, beginning in 1929 and continuing until 1944. Then, and until 1980, no cases were described in the medical literature. Interviews with physicians in practice during this period disclosed only rare cases of BSF. Outbreaks occurred again in Minas Gerais state in 1981, 1984, 1992, 1995, and 2000. Although several cases in these outbreaks were fatal, the diagnoses were not well documented by laboratory methods except during the 1995 and 2000 outbreaks. The true mortality rate of RMSF is often hidden because autopsies are performed in only a low proportion of deaths (5). Immunohistochemical detection of *R. rickettsii* offers an accurate diagnosis both retrospectively in fatal cases and in cutaneous biopsies of lesions during acute illness (6). We describe two outbreaks of BSF in families that occurred in 1995 and 2000 in Novo Cruzeiro and Coronel Fabriciano municipalities of Minas Gerais state, Brazil; six pediatric cases, five fatal, were involved.

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Materials and Methods

Novo Cruzeiro Municipality, located in one of the poorest areas of Minas Gerais state in the northeastern region, has a population of 35,000, who live mainly in rural areas. Coronel Fabriciano Municipality is located in Rio Doce Valley in the eastern part of Minas Gerais state. This region was industrialized 30 years ago, but transition areas with rural characteristics persist in the peripheral area of its cities. Horses are prevalent, a fact that plays an important role in supporting the *Amblyomma cajennense* tick population (Figure 1).

Novo Cruzeiro Municipality

These cases all occurred in the same family in 1995, involving four boys of 9 months, 3 years, 4 years, and 5 years of age. All the patients died, except the 3-year-old boy; a sample of serum collected on day 5 of this boy's illness was tested by indirect immunofluorescence assay (IFA) for antibodies to *R. rickettsii*. A necropsy was performed in the index patient, the 9-month-old boy, and tissue samples of skin, brain, stomach, liver, spleen, and kidney were collected. These materials were fixed in neutral-buffered formaldehyde and shipped to the Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland. Immunohistochemical examination for spotted fever rickettsiae was performed according to the method of Dumler et al. (7).

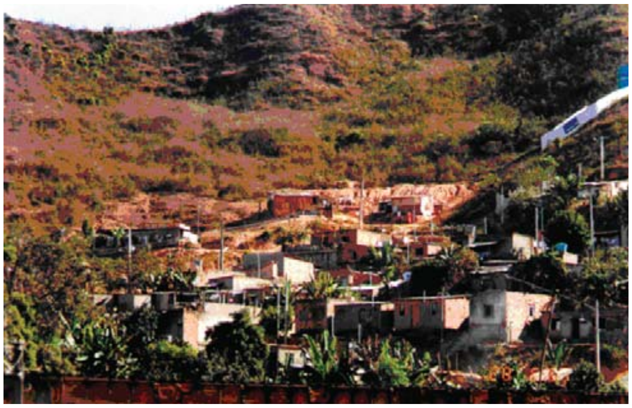

Figure 1. A characteristic habitat in spotted fever–endemic area, Brazil.

Coronel Fabriciano Municipality

Twenty-one suspected cases of BSF in patients with fever and rash were reported in Coronel Fabriciano Municipality during 2000. Thirteen patients had a history of recent tick bite and came from Pedreira, a periurban area with rural characteristics. Among these 21 suspected patients, 2 children (12 and 15 years of age) died. They were brothers who lived in the same house. Fleas, collected from dogs in this house during the outbreak, had *R. felis,* as detected by polymerase chain reaction (PCR) (8). IFAs to detect antibodies to *R. rickettsii, R. typhi*, and *Ehrlichia chaffeensis* were performed on serum from all 21 patients (9). A second serum sample was obtained from nine patients. The reactive serum samples were also tested for antibodies to *R. felis*. A necropsy was performed on the second fatal patient, and samples of skin, brain, stomach, liver, spleen, and kidney were collected. These materials were fixed in neutral-buffered formaldehyde and shipped to the Rickettsial and Ehrlichial Diseases Research Laboratory, Department of Pathology, University of Texas Medical Branch at Galveston, Texas. Immunohistochemical examination for spotted fever rickettsiae was performed by using a monoclonal antibody against a lipopolysaccharide epitope distinctive for spotted fever group rickettsiae (10). PCR was attempted to amplify rickettsial DNA from formalin-fixed, paraffin-embedded necropsy tissues from this patient (11).

Results

In Novo Cruzeiro Municipality, the index patient, a 9-month-old boy, was seen with fever and cough of 5 days' duration; a rash had developed on day 3 of illness. On physical examination, a high fever, maculopapular exanthem, diarrhea, and coma were noted. Four days later, the patient was in hypotensive shock, had a seizure, and died. The other three brothers had fever, nausea, vomiting, and a maculopapular rash. All the brothers had a history of tick bite in the 15 days before the onset of symptoms. The 4- and 5-year-old children died in hypotensive shock on days 8 and 9, respectively, after onset of disease. An IFA antibody titer of 512 against *R. rickettsii* developed in the surviving 3-year-old child 5 days after the onset. A sample of serum collected 6 months later from this patient showed an IFA antibody titer of 8,192 against *R. rickettsii*. Vascular endothelial cells in the liver, stomach, and kidney of the index patient contained spotted fever group rickettsiae demonstrated by immunohistochemical results in multiple foci of lymphohistiocytic vasculitis (Figure 2).

In Coronel Fabriciano Municipality, the first person to become ill was a 12-year-old boy; during the course of disease he had fever, nausea, vomiting, diarrhea, abdominal pain, headache, myalgias, and edema. Before death, stupor and renal failure occurred. Subsequently, fever, rash, nausea, vomiting, diarrhea, abdominal pain, headache, myalgia, jaundice, and renal failure occurred in the 15-year-old brother of the index patient, and he also died. Both patients reported a tick bite. Immunohistochemical evaluation of the necropsy materials obtained in the second fatal case, using an immunoglobulin (Ig) M monoclonal antibody against a lipopolysaccharide epitope specific for *Rickettsia* of the spotted fever group, demonstrated typical rickettsiae in vascular endothelium. Attempted PCR failed to amplify rickettsial DNA from tissues in the case from Coronel Fabriciano, presumably owing to the quality of DNA in the formaldehyde-fixed, paraffin-embedded blocks.

Among 21 suspected cases of spotted fever rickettsiosis in the second half of 2000 in Coronel Fabriciano, serum samples from three patients contained antibodies to *R. rickettsii* detected by IFA in the first sample at a titer of 64, including the serum of one patient who died; 13 of these patients reported tick bites. A second sample of serum was collected for testing for antibodies to *R. rickettsii* in nine of these cases. The second serum sample of one patient, whose first sample was negative, reacted at a titer of 64 on day 12 of disease. Among the three patients whose first

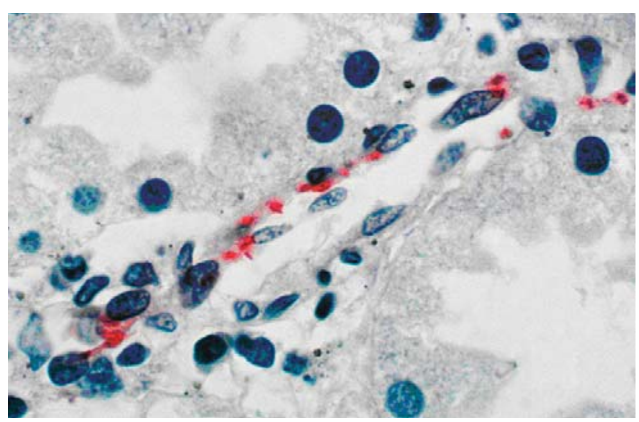

Figure 2. Immunohistochemical stain demonstrates *Rickettsia rickettsii* in endothelial cells of a blood vessel in kidney from patient I. (Hematoxylin counterstain; original magnification X 1200).

samples contained antirickettsial antibodies, a second sample was not collected in two cases, and in one case the titer did not increase when the sample was tested 12 days later. The three patients with IFA antibodies to *R. rickettsii* had fever, headache, and rash. None had IFA antibodies to *R. typhi* or *E. chaffeensis***.** The serum samples that reacted with *R. rickettsii* did not contain antibodies to *R. felis* detected by IFA.

Discussion

R. rickettsii causes the spotted fever rickettsiosis with the highest case-fatality rate. In Brazil, *R. felis* is the only other spotted fever group rickettsia documented to cause human disease (1,12), whereas in the United States rickettsialpox also causes rickettsiosis of this group. Although these diseases are not distinguished by IFA serologic tests, unless cumbersome absorption studies are performed with specific antigens, these five children undoubtedly died of fatal *R. rickettsii* infection.

BSF has been reported in the Brazilian states of Minas Gerais, São Paulo, Rio de Janeiro, Espirito Santo, and Bahia (13), where it is transmitted by *A. cajennense* ticks (14). These ticks are distributed from northern Argentina to southern Texas and could harbor *R. rickettsii* at any location in between. In the United States, *R. rickettsii* is maintained transovarially and transtadially in *Dermacentor* ticks such as *D. andersoni* and *D. variabilis*, although maintenance by means of rickettsemic mammals may also play a major role (15). Only a small fraction, most likely <0.1%, of *A. cajennense* and *D. variabilis* ticks carry *R. rickettsii,* which affects ticks as well as humans (16–18). Thus, investigations of tick populations even in the vicinity of cases of RMSF or BSF do not necessarily detect ticks infected with *R. rickettsii.*

The phenomenon of familial clusters of RMSF has been noted numerous times. In fact, the simultaneous occurrence of severe febrile illness in more than one patient generally suggests person-to-person or a point-source transmission of infection. Few physicians may be aware that 4.4% of cases of RMSF occur in the household of another case-patient with the disease (15), a situation that often lends further diagnostic confusion for this illness that can mimic other febrile exanthems, such as dengue, as well as gastrointestinal infection, other abdominal conditions, pneumonia, and meningoencephalitis (19–22). We realize that some patients with suspected BSF from Coronel Fabriciano Municipality, Brazil, with fever and rash and without seroconversion by IFA to *R. rickettsii,* might have another disease. Dengue fever is also endemic in this region in some periods of the year.

In the United States, the incidence of RMSF undergoes cyclic periods of increase and subsequent decrease extending over decades (23). The consistently rising incidence of RMSF in the United States in 1999 and 2001 suggests that reemergence is occurring. Likewise, these outbreaks of fatal cases in children from Minas Gerais state, Brazil, may indicate a reemergence of BSF in that country. Although we do not have an incidence rate for BSF documented in the 1980s, BSF surveillance was implemented during that period. As a result, an incidence rate of 0.35 BSF cases per 100,000 population has been estimated for the early 1990s (13). A high case-fatality rate of 40% for BSF in Minas Gerais state between 1981 and 1989 also suggests reemergence of this disease.

The mechanisms underlying reemergence and subsidence are not known, but several factors, including suburbanization, destruction of the forests, and increased outdoor activities appear unlikely to be involved. None of these factors decreased markedly during the 1980s and early 1990s, when the incidence of RMSF waned. Now may be an appropriate time to investigate the ecology of *R. rickettsii* as well as to mount a campaign of increased public and physician education regarding RMSF and BSF to avoid deaths from delayed or missed diagnosis of this disease, which is usually difficult to diagnose in its early course. Emphasis should be placed on initiation of therapy with doxycycline in the first 4 days of illness, which dramatically reduces the case-fatality rate of this disease.

Dr. Galvão is currently professor of epidemiology at the Federal University of Ouro Preto. He has a strong interest in spotted fever rickettsiosis in Brazil.

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Divine Mathematics

From Hurricane Sisters

By Ginger Andrews

In her second month of a three-month-long virus,
which, according to half a dozen fellow victims,
does not respond to antibiotics, my sister apologizes
for needing to take her third nap of the day
on my sofa. Homeless and divorced, she's relieved
to know that a trip to the doctor most likely wouldn't
do her any good, especially since she has no insurance
coverage of any kind, except on her '78 Ford Fairmont,
with its brand new master cylinder, which thanks to God
and Les Schwab's low monthly payment plan,
should be paid for by the end of the year,
at which time she hopes to get a rotation,
two new tires, and a badly needed front end alignment,
all for just under a hundred bucks.

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Rapid Antigen-Capture Assay To Detect West Nile Virus in Dead Corvids

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The utility of the VecTest antigen-capture assay to detect West Nile virus (WNV) in field-collected dead corvids was evaluated in Manitoba and Ontario, Canada, in 2001 and 2002. Swabs were taken from the oropharynx, cloaca, or both of 109 American Crows, 31 Blue Jays, 6 Common Ravens, and 4 Black-billed Magpies from Manitoba, and 255 American Crows and 28 Blue Jays from Ontario. The sensitivity and specificity of the antigen-capture assay were greatest for samples from American Crows; oropharyngeal swabs were more sensitive than cloacal swabs, and inter-laboratory variation in the results was minimal. The sensitivity and specificity of the VecTest using oropharyngeal swabs from crows were 83.9% and 93.6%, respectively, for Manitoba samples and 83.3% and 95.8%, respectively, for Ontario birds. The VecTest antigen-capture assay on oropharyngeal secretions from crows is a reliable and rapid diagnostic test that appears suitable for incorporation into a WNV surveillance program.

Since 2000, surveillance for West Nile virus (WNV) in dead corvids has been the cornerstone of the early warning system for this virus in the Canadian public health system. During 2001 and 2002, WNV was detected in avian tissues by using real-time TaqMan reverse transcription–polymerase chain reaction (RT-PCR), as described by Lanciotti et al. (1). Birds collected by local or provincial authorities were sent to regional centers for tissue collection. Tissue samples were shipped daily, often thousands of kilometers, to the National Microbiology Laboratory in Winnipeg, Manitoba, for final diagnosis (by RT-PCR). Though the sensitivity and specificity of TaqMan RT-PCR assay are excellent, the centralization of testing and the multiple steps necessary to extract bird tissues from carcasses, purify RNA, and screen samples for WNV genome presented major logistical, biosafety, reporting, and financial challenges. Komar et al. (2,3) demonstrated that WNV is present in high titer in oral and cloacal cavities of experimentally infected birds. Recently, a rapid antigen-capture wicking assay (VecTest, Medical Analysis Systems, Camarillo, CA) has become commercially available (4,5). Its use for screening swabs from the cloacal or oral cavities of dead birds has yet to be evaluated under field conditions.

The ultimate goal of this study was to determine whether the VecTest assay could serve as a suitable alternative testing procedure for WNV dead bird surveillance. This goal was achieved by quantifying the sensitivity and specificity of this antigen-capture assay to detect WNV in corvids collected as part of routine dead bird surveillance programs in Manitoba and Ontario. The effect of storage conditions and duration of storage of swabs in grinding solution on the sensitivity of the assay and viability of virus was also assessed.

Materials and Methods

Corvids collected as part of the WNV dead bird surveillance programs in Manitoba and Ontario, Canada, in 2001 and 2002 were used. Laboratories in each province received dead birds, collected oropharyngeal or cloacal swabs or tissues, performed the antigen-capture assay, and shipped tissues or swabs to the National Microbiology Laboratory for confirmatory testing. In both laboratories, only birds lacking signs of obvious decay or decomposition were included for testing. Birds typically were shipped to the laboratories via courier. During submission, most animals were held within insulated coolers containing freezer packs. If specimens could not be shipped on the date of collection, most were held at 4°C. On rare occasions, birds were frozen at –20°C by submitters before

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shipment or by the diagnostic laboratories before testing. However, most specimens were held at approximately 4°C for 1 to 4 days before processing.

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Thirty-one American Crows (*Corvus brachyrhynchos*) and 28 Blue Jays (*Cyanocitta cristata*), collected from June to December in 2001 and held at –20°C were tested in March and April 2002. Swabs were taken from the oropharyngeal cavity and cloaca of each bird. In 2002, testing was restricted to crows (n = 222) from which only oropharyngeal swabs were collected and tested by VecTest. Polyester swabs (VWR CanLab, Mississauga, Ontario) were applied to the oropharyngeal cavity and the cloaca. Swabs were placed in microcentrifuge tubes containing 1 mL of BA-1 diluent (similar to that described by Nasci et al. [4]). The antigen-capture assay was carried out according to the manufacturer's instructions except that the aliquot of swab eluate was 125 µL, not 250 µL. To confirm the infection status of birds, approximately 25-mg pieces of brain and kidney from each bird were pooled in microcentrifuge tubes and stored at –80°C before shipment to the National Microbiology Laboratory. At that laboratory, 1 mL of BA-1 diluent and a tungsten bead (QIAGEN, Inc. [Canada], Mississauga, Ontario) were added to each tube, and tissues were homogenized on a Retsech Mixer mill (QIAGEN). After centrifugation, RNA was extracted from 150- to 200-µL aliquots of supernatant.

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Beginning in mid-August, 2002, a total of 109 American Crows, 31 Blue Jays, 6 Common Ravens (*Corvus corax)*, and 4 Black-billed Magpies (*Pica pica*) were examined. Two cloacal swabs (one each from a Common Raven and an American Crow) and one oropharyngeal swab from a Blue Jay were not taken; hence swabs from both sites were available for a total of 147 birds. Specimens were collected by the procedures described above. However, after the 125-µL aliquots of swab eluate were removed, the remainder of the sample was frozen at –80°C and shipped to the National Microbiology Laboratory for confirmatory RT-PCR testing. Tissues were removed and tested for WNV by using RT-PCR on 42 (29 crows, 9 Blue Jays, 3 Common Ravens, and 1 magpie) of the 150 birds.

In both laboratories, swabs were tested for WNV with the VecTest assay (WNV/St. Louis encephalitis antigen panel), as described by Nasci et al. (4), with the following modifications. Swabs were incubated at 37°C for 30 min and shaken intermittently; if debris was present, tubes were centrifuged for 5 min at 4,000 rpm. Aliquots of 125 µL of the manufacturer's grinding solution and of each swab sample were mixed in a microcentrifuge tube. This mixture was centrifuged by using a Vortex mixer (VWR International, Mississauga, Ontario), and test strips were inserted into the supernatant fluid for 15 min, then removed and examined. During this study, two and five different persons from the Manitoba and Ontario laboratories, respectively, interpreted or read the test strips. As illustrated by Nasci et al. (4), presence of WNV was confirmed by the formation of a reddish purple line on the test strip, corresponding to the WNV-positive location and the development of a test control line.

Real-time TaqMan RT-PCR assays to detect WN viral RNA, as described by Lanciotti et al. (1), were conducted on all swabs and representative tissue samples from Manitoba and all tissue samples from Ontario. Briefly, RNA was extracted from 150 µL to 200 µL of swab eluates or tissue homogenates by using QIAamp viral RNA kits (QIAGEN). A final volume of 70 µL of eluted RNA was stored at –80°C until used. Five microliters of RNA was combined with the appropriate primers and probes in buffer with the TaqMan RT-PCR ready-mix kit (PE Applied Biosystems, Foster City, CA). Samples were subjected to 40 amplification cycles in an ABI Prism 7700 Sequence Detection System instrument (PE Applied Biosystems), according to the manufacturer's protocol for TaqMan RT-PCR cycling conditions. All samples were screened with primers and probe specific to the 3′ NTR, and positive extracts were confirmed by reamplification with the ENV primers and probe (1). Swabs or tissues were considered positive only if positive results were obtained with both primer and probe sets. Sensitivity and specificity of the antigen-capture assay applied to oropharyngeal and cloacal swabs from corvids were calculated by using the results of TaqMan RT-PCR assays on swab or tissue samples as the "true" outcome (6).

To establish how storage conditions and duration of storage affected the sensitivity of the antigen-capture assay, cultures of Egypt 101 strain of WNV (approximately 4 x10^7 PFU/mL) were serially diluted to 4 X 10^4 PFU/mL in BA-1 diluent. Polyester swabs were then dipped into each virus solution, placed into 1 mL of manufacturer's grinding solution, and held at –20°C, 4°C, or room temperature (approximately 18°C). The test strips were applied to aliquots of each solution 0, 3, 5, 7, 10, and 14 days later, as described previously. To assess virus viability in grinding solution, 50 µL of each tested grinding solution and virus dilution was placed into 450 µL of BA-1 diluent, and aliquots of 200 µL of this mixture were added to Vero cells reared on standard nutrient medium (i.e., Dulbecco's modified Eagle medium and 10% fetal bovine serum [FBS]) at 37°C and 5% CO_2. After a 1-h adsorption, 2 mL of nutrient medium (same as above but 5% FBS) was added to tissue culture wells, which were incubated as above for up to 7 days. Positive (WNV in BA-1 as above) and negative (media only) controls were maintained under the same conditions, and cells in culture were observed daily for cytopathic effect (CPE). Cells from positive controls and the highest concentration of virus used (i.e., 4 X 10^7 PFU/mL) in grinding solution for each temperature and sampling date were acetone fixed, exposed to WNV-specific antibodies (produced in rabbits and supplied by H. Weingartl, Canadian Food Inspection Agency, Winnipeg, Manitoba) and conjugated goat anti-rabbit immunoglobulin (Ig) G (Kirkegaard & Perry, Gaithersburg, MD) and assessed for fluorescence under a UV microscope.

Results and Discussion

The sensitivity and specificity of the VecTest assay for detecting WNV in oropharyngeal and cloacal swabs are presented in the Table. Data from Ontario reflect the sensitivity and specificity of this antigen-capture assay by using swab eluates in relation to the infection status (TaqMan RT-PCR on kidney and brain) of corvids. In contrast, with the exception of 42 birds in which tissue samples were used to establish WNV infection status, the Manitoba results evaluate the sensitivity and specificity of the antigen-capture assay by using swab eluates in relation to the detection of virus in the same sample by TaqMan RT-PCR. Hence, the results obtained in the two laboratories are not directly comparable.

Data from the Ontario birds from 2001 need to be interpreted with caution since sample size was small and the birds were frozen for up to 10 months. However, cloacal swabs appeared to be less sensitive than oropharyngeal swabs in detecting an infected bird. The sensitivity and specificity of the antigen-capture assay, when oropharyngeal swabs from crows collected in 2002 were used, were 83.3% and 95.8%, respectively, indicating that this assay is acceptable for use as a field detection test in a WNV surveillance program. The advantages of this assay compensate for lower sensitivity only in areas where infected crows are relatively common.

The Manitoba data also indicate that this antigen-capture assay is sufficiently sensitive and specific to detect WNV in oropharyngeal and cloacal swabs from crows when compared to RT-PCR results. Sixty-two of 109 crows were positive by RT-PCR on either oropharyngeal or cloacal swab, although the number of birds that would have been positive using tissues was not determined. All but three birds positive on oropharyngeal swabs were also positive on cloacal swabs. Small sample sizes and the relatively small number of positive samples on RT-PCR make evaluation of the utility of this antigen-capture assay on Blue Jays, Common Ravens, and magpies difficult. In general, the test's specificity seems superior to its sensitivity (Table). Further evaluation is necessary before there can be confidence in this assay as a means of detecting WNV in these species.

In Manitoba, the VecTest assay was not evaluated against RT-PCR on tissue for all birds, and thus the sensitivity and specificity cannot be related to the true infection status of each bird. However, indirect evidence suggests that the Manitoba antigen-capture results approximate those that would have be obtained had RT-PCR on tissues been used in the comparison. On the 29 Manitoba crows in which tissues were used to establish infection status by RT-PCR, the sensitivity and specificity (i.e., 83.3% and 94.1%, respectively) of the antigen-capture assay on oropharyngeal swabs were comparable to those from Ontario. Likewise, the data from Manitoba are similar to the data on sensitivity and specificity of the antigen-capture assay on swab eluates from crows in Ontario and compatible with the high prevalence or titers of virus in oropharyngeal or cloacal samples described by Komar et al. (2,3).

The "false-negative" VecTest samples had higher mean cycle threshold (CT) values (i.e., CT values are a measure of the overall virus titer in samples; higher CT values indicate lower viral loads) for both primer and probe sets (i.e.,

Table. Sensitivity and specificity of the VecTest assay to detect West Nile virus (WNV) in oropharyngeal and cloacal swabs collected from corvids in Ontario and Manitoba

		Oropharyngeal swabs		Cloacal swabs	
Species tested (y)	N	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Ontario					
American Crows (2001)	33	92.8	79	58.3	94.7
Blue Jays (2001)	28	60	100	40	100
American Crows (2002)	222	83.3	95.8	ND[a]	ND
Manitoba					
American Crows	109[b]	83.9	93.6	83.1	97.9
Blue Jays	31[c]	71.4	100	57.1	100
Common Ravens	6[b]	100	100	100	0
Black-billed Magpies	4	0	100	66.7	100

[a]ND, not done.
[b]Cloacal swabs were not available from one bird in each of these groups of birds.
[c]An oropharyngeal swab was not taken from one of the Blue Jays.

26.1± 4.5, generic; ± 26.0± 4.9, envelope) compared to bona fide positives (i.e., 21..0 ± 3.4, generic; 19..7 ± 3.7, envelope). This observation suggests that the viral titers in the false-negative samples were below the threshold for consistent detection when the antigen-capture format was used. Similar but lower levels of sensitivity have been reported for this antigen-capture assay when it has been used to detect WNV in field-collected mosquitoes (4).

Regardless of the storage conditions of swab samples, VecTest strips always detected WNV at viral concentrations >4 X 10^6 PFU/mL but never at titers of 4 x 10^4 PFU/mL. The VecTest assay detected WNV at 4 X 10^5 PFU/mL when the grinding solutions were held at any of the three temperatures for up to 7 days; thereafter WNV was only consistently detected at 4 X 10^5 PFU/mL when solutions were held at –20°C. Grinding solution appears to inactivate WNV. None of the tissue cultures inoculated with grinding solution containing WNV had evidence of CPE or positive indirect fluorescent-antibody assay results, whereas all of the positive controls did. Based on these results, swabs can be placed in grinding solution for up to 7 days before the test strips are applied and held from –20°C to 18°C, without loss of sensitivity. As noted by Nasci et al. (4), once in the grinding solution, the titer of WNV is substantially reduced, as would be any potential biological hazard associated with handling or shipping the swab samples.

In noncorvid species the VecTest assay also has low sensitivity when compared with immunohistochemistry to detect viral antigen in fixed tissue and RT-PCR on frozen tissue. The sensitivity of this antigen-capture assay was 46.7% for oropharyngeal swabs taken from 27 different raptors (of various species) in Ontario during 2002 (G.D. Campbell, Ontario Veterinary College, ON, pers. comm.). This obviates the use of this assay as a reliable screening test for WNV infection in rehabilitation centers, veterinary clinics, zoos, animal disease diagnostic laboratories, and other settings where potential exposure to a biocontainment level 3 agent is of concern.

The advantages of this antigen-capture assay over conventional molecular-based diagnostic procedures such as real-time TaqMan RT-PCR include simplicity of procedures, no requirement for expensive and technically demanding instruments, and much shorter turn-around times for testing (i.e., results are available in 15 min). In addition, testing swabs rather than tissues eliminates the need to dissect submitted birds, thus decreasing the processing time and cost of testing and the risk for lacerations to laboratory workers (7).

The greatest limitation to the use of this system in WNV surveillance programs is the potential loss of information on early season WNV activity. This loss could result from the lower sensitivity of the antigen-capture assay compared to the real-time RT-PCR TaqMan assay. However, the loss of temporal sensitivity likely would be offset somewhat by the rapid turn-around times possible with the antigen-capture format. In addition, in our experience, the VecTest assay does not appear to work as effectively in noncorvid (or all corvid) species. Thus, its usefulness would be greatly diminished in jurisdictions that test all avian species as part of their WNV surveillance programs. The poorer performance of the VecTest assay in noncorvids may be related to species-specific differences (and variability) in virus titers in excretions and secretions at the time of death (3). The VecTest assay will likely replace TaqMan RT-PCR assays as the front-line diagnostic test for future WNV dead bird surveillance programs in Canada. When this antigen-capture assay is used, only index cases of WNV infection in dead birds detected in a given jurisdiction such as a health unit or municipality need to be confirmed with RT-PCR assays before public notification of virus activity. This procedure would compensate for the slightly lower specificity of the antigen-capture assay and should result in markedly decreased workloads for laboratories using molecular diagnostic procedures. VecTest positives obtained thereafter need not be confirmed by an alternative assay since the impact of false positives in a jurisdiction with previously confirmed virus activity should be negligible.

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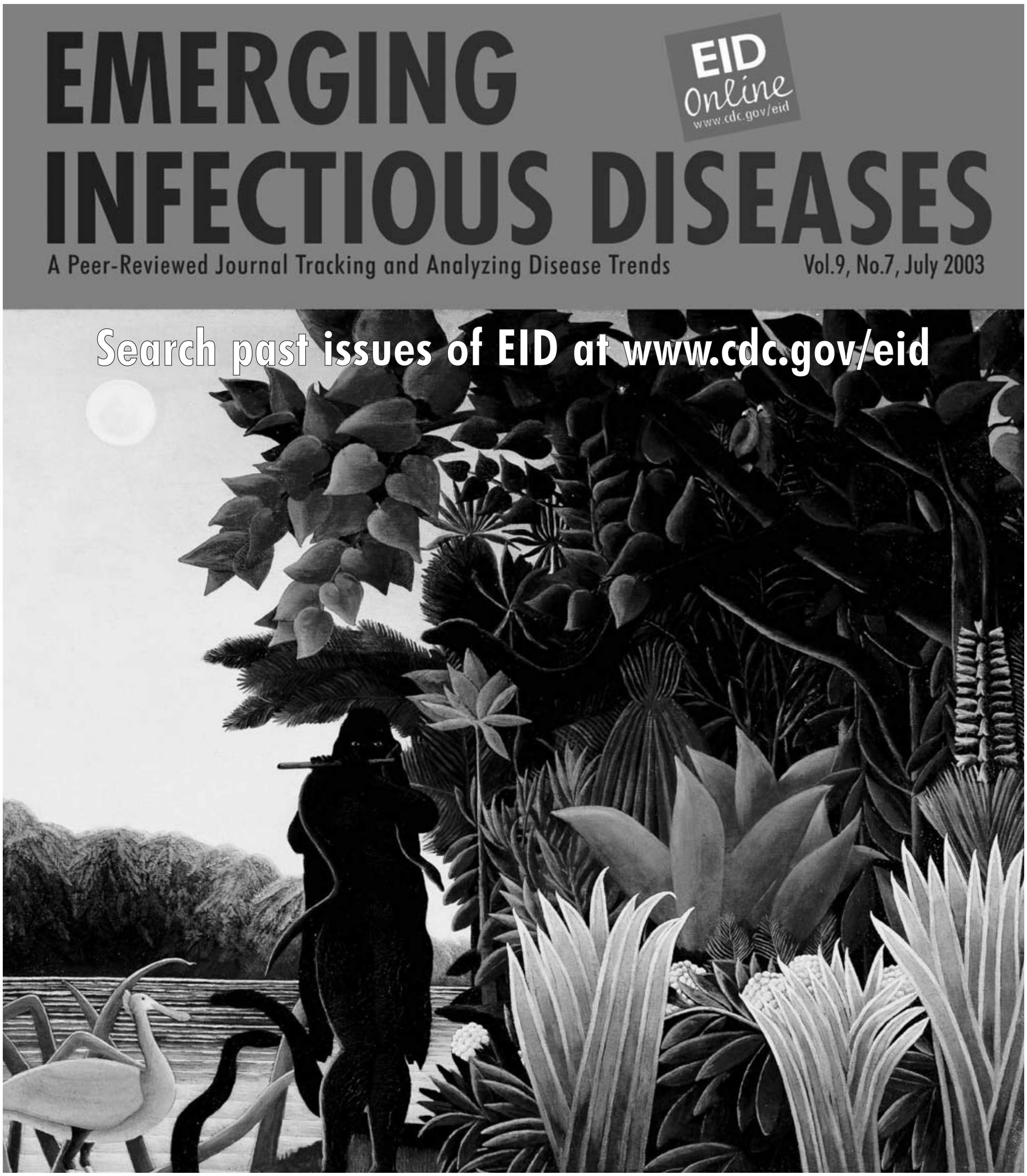

Serologic Evidence of Dengue Infection before Onset of Epidemic, Bangladesh

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Dengue fever emerged in Bangladesh in 2000. We tested 225 serum samples from febrile patients and 184 blood donors in 1996 and 1997 for dengue antibodies; 55 (24.4%) febrile patients had dengue antibodies (65.5% with secondary infection pattern), compared with one (0.54%) donor ($p < 0.001$), suggesting that dengue transmission was ongoing well before 1996.

Dengue continues to spread globally; two fifths of the global population is at risk, primarily within tropical countries (1,2). A proportion of dengue infections result in dengue hemorrhagic fever (DHF), which is associated with high death rates. Most deaths are preventable with timely, careful fluid management. In areas hyperendemic for dengue with clinicians experienced in diagnosis and management of dengue fever and DHF, death rates are relatively low (3,4). Recognition of ongoing dengue transmission is helpful for optimal management and implementation of rational prevention programs (5).

While dengue viruses were likely responsible for what was called Dhaka fever in 1965 (6), dengue fever and DHF were not recognized in recent decades in Bangladesh, until an outbreak occurred in 2000 (7). Nearly 15,000 patients have been hospitalized in Dhaka and other urban areas in Bangladesh since 2000. News reports focus daily on the numbers of new dengue cases, and panic is palpable among residents of Dhaka. We assisted the Government of Bangladesh in designing and implementing emergency strategies to contain the epidemic. Serologic responses of dengue patients (based on evaluating immunoglobulin [Ig] M/IgG ratios) identified during surveillance showed that approximately 70% of patients had also been infected with dengue previously (7), suggesting that unrecognized dengue illnesses had been present.

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The objective of this study was to evaluate whether undiagnosed dengue infection was occurring in Bangladesh before 2000. We studied stored serum samples from a group of febrile patients who attended the Clinical Laboratory of ICDDR,B during 1996 and 1997 and who were evaluated for typhoid.

Materials and Methods

Acute-phase serum specimens, which had been submitted for Widal testing for evaluation of typhoid fever, were identified from 225 febrile patients who attended the Clinical Laboratory of ICDDR,B during 1996 and 1997; specimens were stored at –20°C. We also identified serum samples from 184 blood donors obtained during the same interval and stored under similar conditions. Information about age and sex were not available for blood donors. All 409 serum specimens were tested for antibodies to dengue viruses and Japanese encephalitis virus (JEV) by IgM and IgG antibody–capture enzyme linked immunoassay (8,9).

Microtiter plates were coated with 100 µL goat antihuman IgM and IgG antibodies and incubated at 4°C for 48 to 72 h. Four coated plates were kept at room temperature for half an hour and washed five times with PBS-T (phosphate-buffered saline); 50 µL of diluted patient serum samples and positive and negative controls (1:100) were added into respective wells and incubated at 4°C overnight in a moisture box. After the plates were washed five times with PBS-T, 50 µL pooled antigens of dengue virus (DENV)-1–4 and 50 µL JEV were each added to separate wells and incubated at room temperature for 2 h. After the plates were washed five additional times to remove excess antigens with PBS-T, 25 µL working conjugate was added to each well and incubated at 37°C for 1 h. After the plates were washed with PBS-T five times to remove excess conjugate and PBS x 10 twice, 100 µL ortho-phenylenediamine (OPD) solution was added to each well and incubated at room temperature for 30 min. Finally, 50 µL stop solution (1 M sulfuric acid) was added to each well. An enzyme-linked immunosorbent assay reader measured the optical density (OD) at 492 nm.

OD values were used to calculate binding index and units of IgM and IgG. Binding index was defined as OD of test sample minus OD of negative control divided by OD of weak positive control (defined as 100 U) minus OD of the negative control. We multiplied binding index by 100 to obtain units of respective antibodies. Borderline results (±5 of 40 U) were repeated for validation.

IgM and IgG antibody values of ≥40 U were considered positive for dengue or JEV. When anti-dengue IgM or IgG values were ≥40 U, primary infection (first-time exposure) was defined as a ratio of IgM to IgG ≥1.8 and secondary infection (>1 previous exposure) was defined as a ratio of <1.8 (10).

Widal test was performed by rapid slide titration technique, as described by the manufacturer (Murex Biotech Ltd, Dartford, UK). A single test was performed on all acute-phase serum. A test was defined as positive when the titer was >1:80. However, recognizing the nonspecificity of this break point, we also considered titers of ≥1:320 or fourfold rise in serum antibody between acute- and convalescent-phase serum samples as representing "likely typhoid" (11).

Results

Among 225 febrile patients, 123 (54.7%) were male (Table 1). More than half (52.9%) of the patients were <15 years of age; most of the other patients were young adults <30 years old (32.9%).

Fifty-five (24.4%) febrile patients had dengue antibodies, including 9 with antibodies reacting with JEV antigens; no dengue-negative serum samples reacted with JEV, suggesting that JEV antibody responses represented flavivirus cross-reaction (ratio of anti-dengue IgM units to anti-Japanese encephalitis IgM units were ≥1.0 in all 9) (9). In contrast, among 184 blood donors, one (0.54%) had measurable dengue antibodies ($p < 0.001$ when compared with febrile patients); none had JEV antibodies.

The male (22%) to female (27.5%) proportion of those positive for dengue antibodies was similar (Table 2). Among the 55 febrile patients with evidence of dengue infection, 36 (65.5%) had secondary antibody patterns, and 19 (34.5%) patients had primary patterns. Among those with dengue, secondary pattern was more common in female persons (75%) than in male persons (55.6%; $p = 0.1$). While not statistically significant, children <15 years old were more likely to have a primary pattern (12 [44.4%] of 27) when compared with people ≥15 years old (7 [25%] of 28).

Widal test results were positive (1:80 dilution) in 52 (23.1%) serum samples from febrile patients. Widal test results did not correlate (negatively or positively) with dengue test results; 15 (28.8%) of 52 Widal-positive serum samples had evidence of dengue antibodies compared with 40 (23.1%) of 173 Widal-negative serum samples. When a stricter definition (>1:320 dilution) for a positive Widal test result was used, 3 (16.7%) of 18 positive serum samples had dengue antibodies compared with 52 (25.1%) of 207 negative serum samples ($p > 0.5$).

Table 1. Age and sex distribution of 225 serum samples of febrile patients who attended the clinical laboratory of ICDDR,B, 1996–1997

Age group	Male, no. (%)	Female, no. (%)	Total
<5	21 (17.1)	19 (18.6)	40 (17.8)
5–15	46 (37.4)	33 (32.4)	79 (35.1)
16–29	38 (30.9)	36 (35.3)	74 (32.9)
≥30	18 (14.6)	14(13.7)	32 (14.2)
Total	123 (54.7)[a]	102 (45.3)[a]	225 (100)

[a]Totals represent row percentages; all others are column percentages.

A substantial proportion (47.1%) of febrile patients were seen during July and August (Figure). Among 169 febrile patients during the rainy season and brief postrainy season (May-November, which mirrored the dengue season in Bangladesh during the years 2000 and 2001), 49 (29%) were positive for dengue compared with 6 (10.7%) of 56 febrile patients who were ill during December to April ($p < 0.01$).

Discussion

Except for an epidemic in 1965 (6) and some isolated subsequent reports (12,13), dengue infection was not recognized as an important cause of illness in Bangladesh until 2000. The finding that febrile patients, but not blood donors from Dhaka from the same period, had dengue antibodies suggests that many of the febrile illnesses we evaluated in 1996 were caused by dengue, 4 years before the epidemic dengue was documented. Furthermore, most patients with dengue infection had antibody patterns consistent with previous infection, suggesting that dengue transmission had been ongoing well before 1996. Dengue illness was unrecognized most likely because it often is a self-limited influenzalike illness; more severe forms of dengue are confused with other illnesses prevalent in this tropical, impoverished, and densely populated, developing country.

While febrile patients described in this report were being evaluated for typhoid fever, it appears that they were actually more likely to have dengue. This finding underscores a need for access to diagnostic assays to confirm or broaden clinical suspicion. Diseases like dengue, typhoid, leptospirosis, and influenza, among others, may have signs and symptoms that are clinically indistinguishable. In some circumstances, laboratory confirmation can influence management and clinical outcome for the patient, as well as implementation of public health measures for prevention and control.

Table 2. Distribution of patients positive for dengue primary or secondary antibody response by age and sex

	Positive for dengue, no. (%)[a]				
	Male; n = 27		Female; n = 28		
Age group (y)	Primary	Secondary	Primary	Secondary	Total
<5	1 (33)	2 (67)	0	2(100)	5
5–15	8 (67)	4 (33)	3 (30)	7(70)	22
16–29	2 (33)	4 (67)	2 (18)	9(82)	17
≥30	1 (17)	5 (83)	2 (40)	3(60)	11
Total	12	15	7	21	55

[a]Totals represent row percentages.

The Widal test is an imperfect test for typhoid, though specificity improves somewhat with rising titers (14). We did not observe such increases in specificity for typhoid, based on the proportion of patients with various Widal titers who had dengue antibodies. Some febrile patients may have had nonspecific stimulation of antibodies to O-antigens of enteric bacterial commensals resulting in false positive Widal tests (11). However, the possibility of concomitant infection caused by dengue and typhoid cannot be ruled out for some of these patients, given the exceedingly high incidence of typhoid in this region (15).

A widely held contention is that preexisting antibodies to dengue following previous exposure to the virus may predispose patients to more severe dengue illnesses, such as DHF and dengue shock syndrome because of antibody-dependent enhancement (1). In Bangladesh, thus far, only dengue serotypes 2 and 3 have been identified (7). Our findings suggest that, despite recent recognition of dengue illnesses in Bangladesh, previous exposure is not uncommon. We cannot be certain that DHF was also prevalent well before 2000, since comprehensive medical records needed for retrospective case identification are not available. However, if we assume that the antibody-dependent enhancement-risk hypothesis is correct, earlier dengue transmission within Bangladesh may be responsible for DHF cases now being observed and perhaps represents a substantial risk for greater incidence of DHF in the future, if new dengue serotypes are introduced.

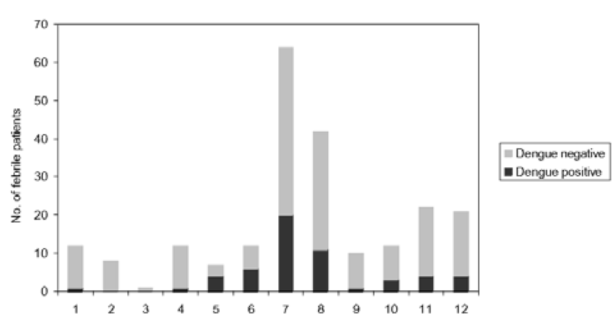

Figure. Distribution of results of dengue serologic testing by months.

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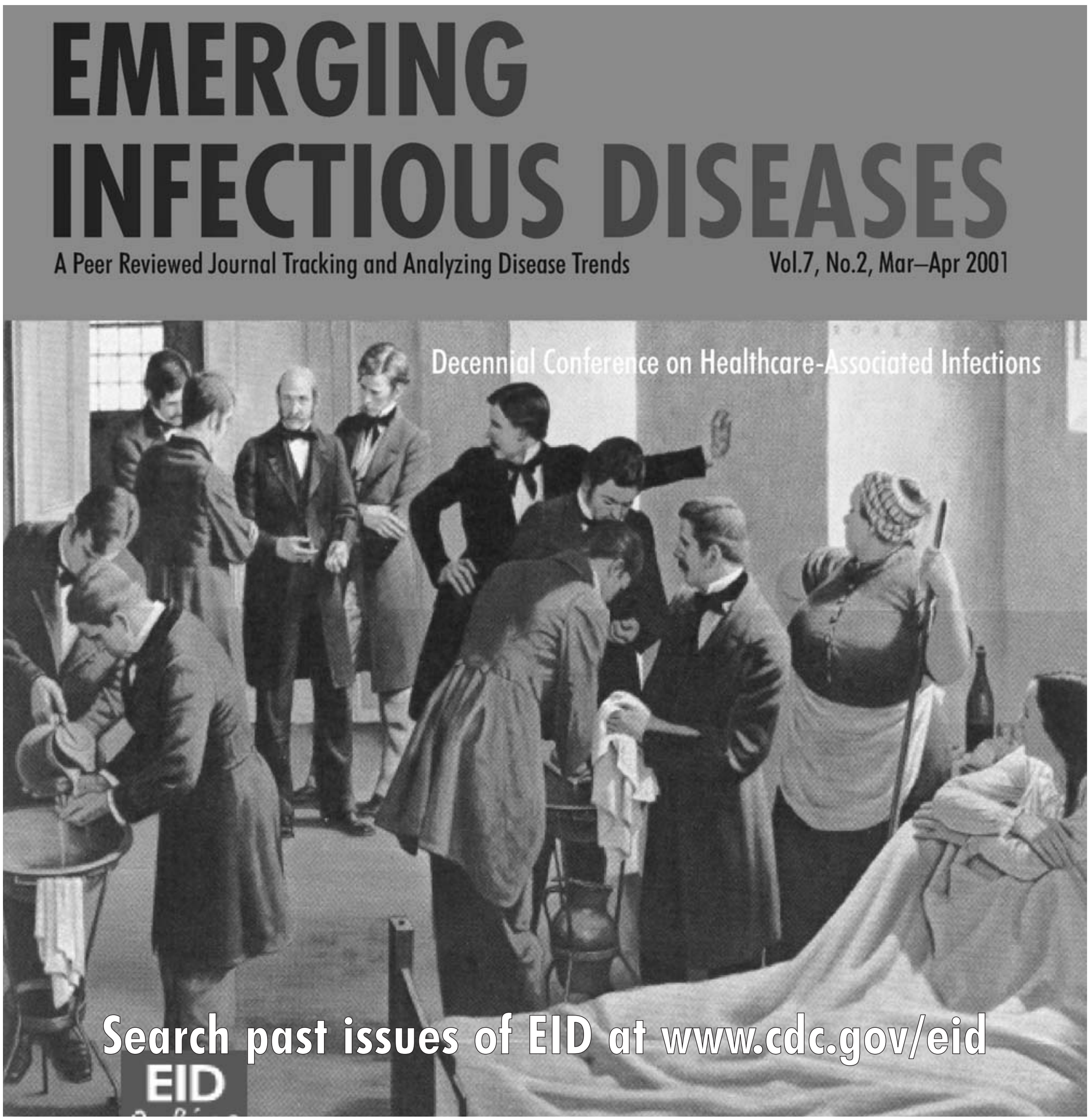

Fluoroquinolones and the Risk for Methicillin-resistant *Staphylococcus aureus* in Hospitalized Patients[1]

Stephen G. Weber,* Howard S. Gold,* David C. Hooper,† A.W. Karchmer,* and Yehuda Carmeli*

To determine whether fluoroquinolone exposure is a risk factor for the isolation of *Staphylococcus aureus* and whether the effect is different for methicillin-resistant *S. aureus* (MRSA) versus methicillin-susceptible *S. aureus* (MSSA), we studied two case groups. The first case group included 222 patients with nosocomially acquired MRSA. The second case group included 163 patients with nosocomially acquired MSSA. A total of 343 patients admitted concurrently served as controls. Outcome measures were the adjusted odds ratio (OR) for isolation of MRSA and MSSA after fluoroquinolone exposure. Exposure to both levofloxacin (OR 5.4; $p < 0.0001$) and ciprofloxacin (OR 2.2; $p < 0.003$) was associated with isolation of MRSA but not MSSA. After adjustment for multiple variables, both drugs remained risk factors for MRSA (levofloxacin OR 3.4; $p < 0.0001$; ciprofloxacin OR 2.5; $p = 0.005$) but not MSSA. Exposure to levofloxacin or ciprofloxacin is a significant risk factor for the isolation of MRSA, but not MSSA.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been implicated as a pathogen in hospital-acquired infections since the 1960s (1). During the 1990s, the proportion of nosocomial infections caused by MRSA increased substantially, and MRSA is now a leading cause of such infections in the United States (2). According to data from the SENTRY Antimicrobial Surveillance Program, approximately 40% of *S. aureus* isolates recovered in intensive care units (ICU) are resistant to methicillin (3). Recently, MRSA infections acquired in the community have been identified as emerging pathogens responsible for substantial disease and death (4,5). While no satisfactory explanation exists for the recent proliferation of MRSA, expanded use of antimicrobial drugs in sites outside the hospital has been suggested as a major contributor to emerging resistance in the community (6).

Fluoroquinolones are among the most commonly prescribed classes of antimicrobial drugs in both the hospital and in the community. Ciprofloxacin, one of the first fluoroquinolones to gain extensive clinical use, was originally heralded for its activity against a broad range of pathogens, including MRSA (7). However, by the early 1990s, many MRSA isolates from clinical specimens were found to be resistant to ciprofloxacin (8). The next generation of fluoroquinolones, including levofloxacin, was introduced during the second part of the 1990s and promised improved activity against gram-positive pathogens. Unfortunately, screening of large numbers of staphylococcal bloodstream isolates as part of the SENTRY Antimicrobial Surveillance Program demonstrated resistance to many of the newest fluoroquinolones as well (9).

Several recent investigations offer preliminary evidence that suggests that the fluoroquinolones themselves may actually predispose patients to infection with or carriage of MRSA. A comparison of microbiology laboratory data with antimicrobial reimbursement reports found a significant correlation between ciprofloxacin prescriptions and the isolation of MRSA (10). Two case-control studies examining risk factors for MRSA have found a significant association between fluoroquinolone exposure and MRSA isolation or infection (11,12). Preliminary analysis from one also suggested a difference in risk between members of the class. Additionally, a prospective study examining the impact of nasally administered mupirocin ointment on MRSA carriage also identified fluoroquinolone exposure as a risk factor for MRSA carriage (13). However, none of these studies was designed specifically to examine the risk associated with fluoroquinolones. Moreover, the design of the prior case-control investigations, as a result of the inappropriate use of patients colonized or infected with sensitive strains for controls, may have yielded biased results.

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[1]This study was presented in part at the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, California, September 2002.

Thus, the association between fluoroquinolone exposure and MRSA remains to be confirmed.

This study was specifically designed to determine whether exposure to fluoroquinolones is a risk factor for the subsequent isolation of *S. aureus*, and whether the effect is different for MRSA versus methicillin-susceptible *S. aureus* (MSSA). In addition, we sought to preliminarily explore any difference in risk between levofloxacin and ciprofloxacin.

Methods

The study was performed at the Beth Israel Deaconess Medical Center, a 640-bed tertiary care teaching hospital in Boston, Massachusetts. The case-case-control method was used. As originally described by Kaye et al., this technique more rigorously upholds the epidemiologic standard that members of a control group be selected independently of their exposure status (14,15). A number of subsequent studies and commentary have upheld the utility of this method as providing the most accurate estimates of risk in studies of antimicrobial drug resistance (15–21). The case-case-control design is actually composed of two parallel case-control studies. Here, the first group of cases consisted of patients from whom nosocomially acquired MRSA was isolated. The second case group was comprised of patients from whom nosocomially acquired MSSA was recovered. *S. aureus* isolates were identified by using standard laboratory procedures. Resistance was determined according to the National Committee for Clinical Laboratory Standards guidelines with automated microdilution testing with the VITEK 2 system (bioMérieux, Hazelwood, MO). In addition, oxacillin resistance was confirmed on MRSA screening agar. A microbiology laboratory database of clinical cultures was searched to identify patients from whom *S. aureus* was isolated from November 1, 1999, to August 1, 2001. Any patient from whom *S. aureus* was first recovered during the initial 72 hours after admission to the medical, surgical, or obstetric services was presumed to have acquired the organism before hospitalization and was excluded. The control group for each of the two component studies was composed of a computer-generated random sample of patients admitted during the same period to the medical, surgical, or obstetric services from whom *S. aureus* was not isolated. To be included, control patients needed to be hospitalized for at least 72 hours. The same group of control patients was used for both cases with MRSA and MSSA.

Data regarding candidate risk factors were collected from existing administrative, pharmacy, and laboratory databases by using a relational database management system (Access; Microsoft Corporation, Redmond, WA). In addition to patient sex and age, coexisting medical conditions were analyzed; these included the presence or absence of cardiovascular, lung, hepatic or renal disease; previous organ transplant; AIDS; malignancy; and diabetes mellitus. Factors specifically relating to hospitalization were collected and analyzed, including transfer from another hospital or care facility, prior surgical procedure or ICU stay, presence or absence of intravenous line, emergent admission, admission service (medicine, surgery, or obstetrics), and the number of days at risk for infection. For case-patients, the last was equal to the number of days of hospitalization before the first isolation of *S. aureus*. For control patients, the number of days at risk was defined as the total length of stay. While the primary objective was to measure the specific risk associated with exposure to levofloxacin or ciprofloxacin, also considered was exposure to a number of other antimicrobial agents, including vancomycin, penicillins, β-lactam/β-lactamase inhibitor combinations, first- and second-generation cephalosporins, third-generation cephalosporins, carbapenems, clindamycin, and metronidazole. During the study period, levofloxacin and ciprofloxacin were the only fluoroquinolones used routinely at the Beth Israel Deaconess Medical Center. To be included as an exposure, any antimicrobial drug dosing, surgical procedure, ICU stay, and placement of intravenous line had to occur before the isolation of *S. aureus* in case-patients.

According to the hospital's infection-control policy, all patients from whom MRSA is isolated are placed on contact precautions in a private room. In addition, patients from whom resistant bacteria have been isolated during prior hospitalizations are automatically flagged and isolated at the time of readmission to minimize cross-contamination. Appropriate adherence to these policies by the staff is consistent across different care units. During the study period, patients diagnosed with MRSA infection were treated with vancomycin.

The SAS software package (SAS Institute, Cary, NC) was used for all statistical analysis. Because the opportunity for both exposures and outcome (i.e., a clinical culture positive for *S. aureus*) would necessarily increase with the number of days at risk, simple univariate analysis does not appropriately reflect the individual risk associated with each of the candidate risk factors. Therefore, a two-variable logistic regression model adjusted for time at risk was used to specify the individual risk associated with levofloxacin, ciprofloxacin, and each of the other candidate risk factors. Odds ratios (OR), 95% confidence intervals (CI), and p values were calculated for each.

To quantify more accurately the specific risk for *S. aureus* isolation after exposure to levofloxacin or ciprofloxacin, variables with a p value of <0.05 in the adjusted univariate analysis were included in a logistic regression model along with each of the fluoroquinolones. Separate models were constructed for MRSA and MSSA cases. Automated selection functions were not used. Candidate risk factors that reached statistical significance ($p < 0.05$) were retained in the multivariable model to adjust the risk associated with levofloxacin and ciprofloxacin. For each of the two case groups, candidate risk factors that did not reach statistical significance in the multivariable model were only allowed to remain in the model as confounders if their removal changed the coefficient for levofloxacin or ciprofloxacin by $\geq$10%. Interaction terms for each of the risk factors included in the final model were similarly included if these criteria were met. Each of the final multivariable models was tested for overfitting by using the bootstrap method (1,000 bootstrap samples of all of the data). Goodness-of-fit of the final models was evaluated with the Hosmer-Lemeshow test. The project was approved by the institutional review board of the Beth Israel Deaconess Medical Center

Results

Two hundred twenty-two patients with nosocomial MRSA and 163 with nosocomial MSSA were identified and served as the two case groups. For both, 343 randomly selected inpatients hospitalized for at least 72 hours were identified as controls. The mean age of MRSA and MSSA cases was 66.2 and 63.3 years, respectively. Control patients, with a mean age of 57.6 years, were significantly younger. Compared to 39.9% of controls, 56.8% of MRSA patients and 55.8% of MSSA patients were men. The mean number of days in hospital before the first positive culture was 12.4 and 7.7 for MRSA and MSSA patients, respectively. In comparison, the number of days at risk for control patients was 6.7 days, significantly shorter than for those from whom resistant organisms were isolated. In total, 67.6% of the MRSA patients were exposed to one of the two fluoroquinolones under study. This value was significantly greater than the number for MSSA (22.7%) or control (21.0%) patients. Of all MRSA isolated, 97% were resistant to fluoroquinolones as opposed to 8% of MSSA strains. As described elsewhere, approximately 80% of isolates tested were variants of a single pulsed-field gel electrophoresis type (22). In addition, during the study, no epidemiologically related outbreak of *S. aureus* disease was detected at the institution.

MRSA Versus Control Patients

The results of univariate analyses for MRSA adjusted for days at risk are shown in Table 1. Exposure to both levofloxacin (OR 5.4; $p < 0.0001$) and ciprofloxacin (OR 2.2; $p = 0.0027$) was more common among MRSA case-patients than controls. In addition, male patients were more likely to be case-patients than controls (OR = 1.8; $p = 0.014$). When compared with patients $\leq$50 years of age, those 50–75 years of age (OR = 2.4; $p < 0.0001$) and >75 years of age (OR = 2.9; $p < 0.001$) were more likely to be cases. Cardiovascular (OR 1.8; $p = 0.0043$), lung (OR 6.8; $p < 0.0001$), renal (OR 2.0; $p = 0.0114$), and hepatic (OR 2.4; $p = 0.0107$) disease were all more common among cases than controls. Hospital transfer (OR 2.9; $p < 0.0001$), ICU stay (OR 7.7; $p < 0.0001$), intravenous line (OR 2.1; $p = 0.0008$), and emergency room admission (OR 2.2; $p < 0.0001$) were also significantly more frequent among case-patients than controls. Patients admitted to the obstetrics service, when compared with those admitted to the medical service, were more likely to be controls than case-patients (OR 0.1; $p < 0.0001$). In addition to the fluoroquinolones, exposure to several other antimicrobial drugs was significantly more common among cases than controls, including vancomycin (OR 4.0; $p < 0.0001$), penicillin (OR 2.1; $p = 0.0042$), third-generation cephalosporins (OR 3.7; $p < 0.0001$), clindamycin (OR 5.4; $p < 0.0001$), and metronidazole (OR 4.2; $p < 0.0001$).

Multivariable models to quantify the specific risk associated with levofloxacin or ciprofloxacin were constructed for MRSA cases. The results are shown in Table 2. After adjustment for other significant and confounding variables, including other antibiotic exposures, exposure to either levofloxacin (OR 3.4; $p = 0.0001$) or ciprofloxacin (OR 2.5; $p = 0.0049$) was more common among MRSA cases than controls. No interaction terms met the significance criteria for inclusion in the final model.

MSSA Versus Control Patients

The results of univariate analyses adjusted for days at risk for MSSA are shown in Table 3. As opposed to the situation for those with MRSA, MSSA cases were no more likely than controls to have been exposed to levofloxacin or ciprofloxacin. MSSA case-patients were more likely than controls to be male (OR 1.9; $p = 0.0011$). Patients >75 years old were more likely than those $\leq$50 to be case-patients (OR 2.1; $p = 0.0037$). Some coexisting conditions were more common among case-patients than controls, including cardiovascular (OR 2.1; $p = 0.0004$), lung (OR 3.0; $p < 0.0001$), renal (OR 2.1; $p = 0.0119$), and hepatic disease (OR 3.2; $p = 0.0006$). Hospital transfer (OR 2.1; $p = 0.0076$), ICU stay (OR 6.4; $p < 0.0001$), intravenous line (OR 2.0; $p = 0.0023$), and emergency room admission (OR 2.0; $p = 0.0009$) were also more likely among case-patients than controls. When compared with patients admitted to the medical service, those admitted to the surgical service were more likely to be cases (OR 1.5; $p = 0.0497$), and those admitted to obstetrics were more likely to be controls (OR 0.1; $p = 0.0002$). Among antimicrobial agents, only exposure to penicillin (OR 2.2; $p = 0.0039$) and metronidazole (OR 1.7; $p = 0.043$) was more frequent among cases than controls.

The results for the multivariable models quantifying the specific risk for MSSA associated with levofloxacin or

Table 1. Results of univariate analysis for MRSA cases and controls

Risk factor	Uncolonized (n = 343) no.	Uncolonized (n = 343) %	MRSA cases (n = 222) no.	MRSA cases (n = 222) %	OR (95% CI)	p value
Demographic						
Male	137	39.9	126	56.8	1.84 (1.27 to 2.67)	0.001
Age	mean = 57.6 y		mean = 66.2 y			<0.001
≤50	131	38.2	39	17.6	—	—
51–75	137	39.9	110	49.5	2.39 (1.50 to 3.83)	<0.001
>75	75	21.9	73	32.9	2.86 (1.70 to 4.79)	<0.001
Coexisting condition						
Cardiovascular disease	186	54.2	156	70.3	1.77 (1.20 to 2.31)	0.004
Lung disease	57	16.6	130	58.6	6.74 (4.43 to 10.26)	<0.001
Renal disease	30	8.7	46	20.7	2.02 (1.17 to 3.47)	0.01
Hepatic disease	17	5.0	28	12.6	2.43 (1.23 to 4.79)	0.01
Organ transplant	4	1.2	2	0.9	0.52 (0.08 to 3.36)	0.49
AIDS	5	1.5	4	1.8	1.25 (0.30 to 5.14)	0.76
Malignancy	54	15.7	32	14.4	0.98 (0.59 to 1.64)	0.94
Diabetes mellitus	67	19.5	56	25.2	1.26 (0.81 to 1.96)	0.31
Hospital factors						
Transfer	32	9.3	53	23.9	2.88 (1.71 to 4.83)	<0.001
Surgical procedure	148	43.1	96	43.2	0.74 (0.51 to 1.09)	0.12
ICU stay	54	15.7	144	64.9	7.66 (5.01 to 11.71)	<0.001
Intravenous line	59	17.2	80	36.0	2.06 (1.38 to 3.16)	<0.001
Emergent admission	149	43.4	136	61.3	2.20 (1.51 to 3.21)	<0.001
Admission service						
Medical	174	50.7	154	69.4	—	—
Obstetrical	62	18.1	2	0.9	0.05 (0.01 to 0.21)	<0.001
Surgical	107	31.2	66	29.7	0.74 (0.49 to 1.10)	0.14
Antimicrobial drugs						
Any fluoroquinolone	72	21.0	150	67.6	5.41 (3.60 to 8.11)	<0.001
Levofloxacin	42	12.2	109	49.1	5.36 (3.45 to 8.32)	<0.001
Ciprofloxacin	34	9.9	62	27.9	2.16 (1.31 to 3.56)	0.002
Vancomycin	36	10.5	94	42.3	3.98 (2.49 to 6.34)	<0.001
Penicillin	36	10.5	56	25.2	2.08 (1.26 to 3.45)	0.004
β-lactam and inhibitor	5	1.5	13	5.9	2.74 (0.88 to 8.46)	0.08
First-generation cephalosporin	77	22.4	45	20.3	0.77 (0.49 to 1.21)	0.25
Third-generation cephalosporin	28	8.2	74	33.3	3.66 (2.18 to 6.13)	<0.001
Carbapenem	3	0.9	7	3.2	1.63 (0.37 to 7.10)	0.52
Clindamycin	9	2.6	32	14.4	5.36 (2.39 to 12.01)	<0.001
Metronidazole	42	12.2	104	46.8	4.20 (2.70 to 6.55)	<0.001

[a]All results adjusted for time at risk. MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odds ratio; CI, confidence interval; ICU, intensive care unit.

ciprofloxacin after adjustment for other factors are shown in Table 2. In contrast to the findings for MRSA, MSSA case-patients were not significantly more likely to have previously received levofloxacin (OR 0.7; p = 0.3023) and tended to have a smaller risk of having received ciprofloxacin (OR 0.5; p = 0.0571) than the controls.

Effects of Fluoroquinolone Exposure on MRSA and MSSA

In this study, patients from whom nosocomially acquired MRSA was isolated were approximately three times as likely as those with MSSA to have received prior therapy with levofloxacin or ciprofloxacin (67.6% vs. 22.7%). Adjusting for time at risk, MRSA isolation and prior exposure to both levofloxacin (OR 5.4; p < 0.0001) and ciprofloxacin (OR 2.2; p = 0.0027) were associated. For MSSA, the association was not significant for either levofloxacin (OR 1.1; p = 0.77) or ciprofloxacin (OR 0.74; p = 0.37). After adjusting for multiple risk factors, including exposure to other antimicrobial classes, exposures to levofloxacin (OR 3.4; p < 0.0001) and to somewhat lesser degree ciprofloxacin (OR 2.5; p = 0.0049) were significantly associated with MRSA. For MSSA cases, exposure tended to be protective for ciprofloxacin (OR 0.5; p = 0.0571), but not for levofloxacin (OR 0.7; p = 0.3023) (Figure).

Discussion

This case-case control study is the first specifically designed to examine the epidemiologic link between fluoroquinolone exposure and the subsequent isolation of *S. aureus* and to specifically differentiate between MRSA and MSSA. After controlling for possible confounders, including exposure to other antimicrobial agents, the results reported here demonstrate a highly significant asso-

Table 2. Results of multivariable analysis

Risk factor	MRSA cases		MSSA cases	
	OR (95% CI)	p value	OR (95% CI)	p value
Primary covariates				
Levofloxacin	3.38 (1.94 to 5.90)	<0.001	0.69 (0.34 to 1.40)	0.30
Ciprofloxacin	2.48 (1.32 to 4.67)	0.005	0.47 (0.21 to 1.02)	0.06
Other covariates				
Lung disease	3.94 (2.43to6.40)	<0.001	2.33 (1.43 to 3.81)	<0.001
Renal disease	*		1.98 (1.03 to 3.80)	0.04
Penicillin	*		1.78 (0.93 to 3.39)	0.08
Metronidazole	1.92 (1.10 to 3.37)	0.02	1.29 (0.65 to 2.56)	0.46
ICU stay	5.33 (3.28 to 8.68)	<0.001	4.60 (2.90 to 7.30)	<0.001
Emergent admission	1.74 (1.09 to 2.78)	0.02	1.90 (1.17 to 3.08)	0.01
Admission service				
Medical	*		—	—
Obstetrical	*		0.29 (0.08 to 1.05)	0.06
Surgical	*		1.82 (1.12 to 2.97)	0.02

[a]All results adjusted for time at risk. Only variables significant ($p \leq 0.05$) on univariate analysis were included in final models. Several variables (indicated with an asterisk) met criteria for inclusion in the MSSA (methicillin-susceptible *Staphylococcus aureus*), but not the MRSA (methicillin-resistant *Staphylococcus aureus*) model. OR, odds ratio; CI, confidence interval; ICU, intensive care unit.

ciation between treatment with levofloxacin or ciprofloxacin and subsequent isolation of MRSA, but not MSSA. The magnitude of the risk is less than reported in earlier studies that did not examine this specific question and whose design would tend to bias results.

Substantial variability exists between both case groups and controls with respect to a number of characteristics, including coexisting illnesses, days spent in intensive care, and presence of intravascular catheters. However, such differences, several of which reflect variation in severity of illness and immune status between the groups, do not negatively affect the interpretation of the results. In fact, such dissimilarity, familiar to clinicians, mirrors the very real differences that exist between colonized or infected and uncolonized patients. Because the same control group is employed for both MRSA and MSSA patients, the case-case-control method accounts for any potential confounding attributable to these differences. Moreover, the sustained association between fluoroquinolone exposure and MRSA after adjusting for these surrogates of disease severity and host immune status in the multivariable model further supports the relationship. This conclusion was tested in the handling of patients from the obstetrics service. A priori, we elected to include both cases and controls from the obstetric service. However since this patient population contributed a significantly larger proportion to the control group (18.1%) than to each of the case groups (0.9% and 1.8%), we performed a subgroup analysis to confirm the aforementioned interpretation. When the subgroup analysis was performed excluding patients from the obstetrics service, the overall results of the study were not significantly changed.

Even in the face of intense selection pressure from exposure to antimicrobial drugs, MSSA isolates very rarely develop methicillin resistance. Therefore, any relationship between fluoroquinolones and MRSA probably occurs at the level of host colonization. With this in mind, the findings could be attributed to a number of etiologic mechanisms.

By eradicating the generally susceptible microorganisms that colonize the skin and mucus membranes (e.g., nares, perirectal area), fluoroquinolone exposure effectively opens an ecologic niche, rendering an inpatient vulnerable to subsequent colonization and infection by the more resistant strains endemic in the hospital, including MRSA. Because fluoroquinolone resistance is relatively rare among strains of MSSA while MRSA isolates tend to be resistant (23), the net result could be the replacement of MSSA with MRSA after fluoroquinolone exposure.

Using in vitro analysis, Venezia et al. performed population analysis on fluoroquinolone-susceptible, *mecA*-positive methicillin-heteroresistant strains of *S. aureus*. Growth in the presence of 0.5 MIC of a fluoroquinolone (ciprofloxacin, levofloxacin, moxifloxacin, or gatifloxacin) resulted in a >10-fold increase in the proportion of the population that grew on high concentrations of oxacillin. The increase was directly proportional to the concentration of the fluoroquinolone and could be detected within 8 hours of exposure. The authors conclude that fluoroquinolones might influence oxacillin resistance by selective inhibition or killing more susceptible subpopulations of heteroresistant *S. aureus*. The surviving subpopulations are more resistant to both oxacillin and the quinolones (24).

While plausible, neither of the first two explanations is completely supported by the results of our study. Were the relationship between fluoroquinolones and MRSA solely attributable to selective pressure that favors the acquisi-

Table 3. Results of univariate analysis for MSSA cases

Risk factor	MSSA cases (n = 163)			
Demographic	No.	%	OR (95% CI)	p value
Male	91	55.8	1.89 (1.29 to 2.77)	0.001
Age	Mean = 63.3 y.			
≤50	42	25.8	—	—
51–75	68	41.7	1.50 (0.95 to 2.37)	0.08
>75	53	32.5	2.10 (1.27 to 3.45)	0.004
Coexisting condition				
Cardiovascular disease	117	71.8	2.08 (1.39 to 3.13)	<0.001
Lung disease	61	37.4	2.95 (1.92 to 4.54)	<0.001
Renal disease	28	17.2	2.06 (1.17 to 3.62)	0.01
Hepatic disease	24	14.7	3.16 (1.64 to 6.10)	<0.001
Organ transplant	2	1.2	1.00 (0.18 to 5.60)	0.99
AIDS	1	0.6	0.39 (0.00 to 3.42)	0.40
Malignancy	30	18.4	1.21 (0.74 to 1.98)	0.46
Diabetes mellitus	38	23.3	1.25 (0.80 to 1.98)	0.33
Hospital factors				
Transfer	29	17.8	2.11 (1.22 to 3.64)	0.01
Surgical procedure	62	38.0	0.78 (0.52 to 1.15)	0.20
ICU stay	89	54.6	6.38 (4.14 to 9.83)	<0.001
Intravenous line	50	30.7	1.99 (1.28 to 3.09)	0.002
Emergent admission	97	59.5	1.90 (1.30 to 2.79)	<0.001
Admission service				
Medical	85	52.1	—	—
Obstetrical	3	1.8	0.10 (0.03 to 0.34)	<0.001
Surgical	75	46.0	1.49 (1.00 to 2.21)	0.05
Antimicrobial drugs				
Any fluoroquinolone	37	22.7	0.96 (0.60 to 1.53)	0.86
Levofloxacin	24	14.7	1.09 (0.62 to 1.90)	0.77
Ciprofloxacin	14	8.6	0.74 (0.38 to 1.44)	0.37
Vancomycin	31	19.0	1.78 (1.04 to 3.05)	0.36
Penicillin	33	20.2	2.17 (1.28 to 3.66)	0.004
β-lactam and inhibitor	6	3.7	2.37 (0.70 to 8.06)	0.17
First-generation cephalosporin	27	16.6	0.65 (0.40 to 1.07)	0.09
Third-generation cephalosporin	18	11.0	1.21 (0.64 to 2.30)	0.56
Carbapenem	2	1.2	1.05 (0.17 to 6.48)	0.96
Clindamycin	6	3.7	1.35 (0.47 to 3.91)	0.58
Metronidazole	34	20.9	1.70 (1.02 to 2.83)	0.04

[a] MSSA, methicillin-susceptible *Staphylococcus aureus*; OR, odds ratio; CI, confidence interval.

tion or emergence of fluoroquinolone-resistant strains, the same phenomenon would be expected to apply with other antimicrobial agents to which MRSA are also resistant. For example, first-generation cephalosporins have activity against most strains of MSSA but nearly all MRSA isolates are resistant. Nevertheless, first-generation cephalosporins were not identified as a unique risk factor for either organism in this study. The same holds true for exposure to clindamycin.

We think that these results support a third mechanism that is independent of the specific antimicrobial agent activity of the fluoroquinolones. Bisognano et al. have suggested an alternative mechanism by which the fluoroquinolones could uniquely predispose to colonization (and subsequent infection) with *S. aureus*. In a series of in vitro experiments, these researchers have demonstrated that exposure to subinhibitory levels of ciprofloxacin results in increased expression of adherence factors promoting host colonization. Isogenic *S. aureus* mutants expressing varying levels of fluoroquinolone resistance, when grown in the absence of drug, showed little difference in adhesion characteristics when compared with parental strains. However, impressive changes in adhesion were exhibited when strains were grown in the presence of 1/4 MIC of ciprofloxacin. This increased adhesion, which was most pronounced among highly resistant mutants, occurred at therapeutically achievable concentrations of ciprofloxacin and was associated with overexpression of fibronectin-binding protein (25). In subsequent work with clinical specimens, the same group showed that 8 of 10 MRSA isolates and 4 of 6 MSSA isolates exhibited increased attachment to fibronectin-coated surfaces after growth in the presence of subinhibitory concentrations of ciprofloxacin. Further, they demonstrated that the effect is mediated at the level of transcription by activation of the *fnb* promoter (26). More recently, the same group showed that the SOS

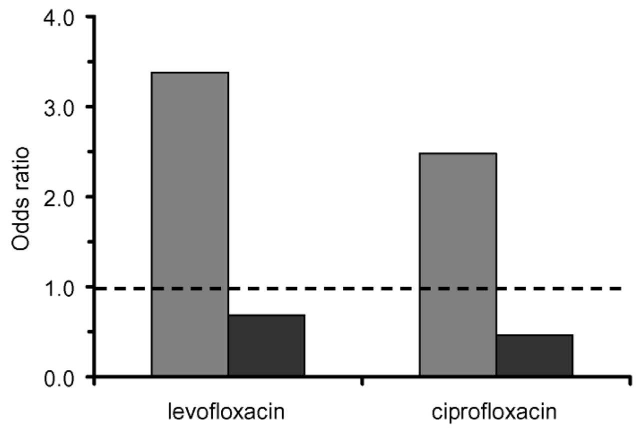

Figure. Odds ratios from multivariable analysis for the isolation of MRSA (methicillin-resistant *Staphylococcus aureus*) and MSSA (methicillin-susceptible *Staphylococcus aureus*) after exposure to levofloxacin or ciprofloxacin. Results for MRSA shown in gray and for MSSA in black. All results adjusted for time at risk.

RecA-mediated pathway, but not the *agr* or *sar* regulatory elements, plays a role in the control of this phenomenon (27,28).

While the association between levofloxacin or ciprofloxacin exposure and MRSA colonization could be at least partially explained by the promotion of *S. aureus* adherence, the present epidemiologic study suggests a means by which the phenomenon would apply whether or not the upregulation of fibronectin binding is a feature unique to resistant isolates. Exposure to levofloxacin or ciprofloxacin that promotes increased adherence of both MRSA and MSSA would have the net effect of increasing the likelihood of recovery of fluoroquinolone-resistant isolates. Because most MSSA isolates, as opposed to MRSA, are susceptible to the fluoroquinolones (92% vs. 3% in this study), MSSA would be selectively eliminated by the antimicrobial agent action of either drug before colonization could be established. In summary, fluoroquinolone exposure would have the dual effect of promoting *S. aureus* colonization while selectively eradicating MSSA strains; the net effect of which is to favor MRSA colonization.

In addition to the overall effect of the fluoroquinolones, we also sought to examine the difference in risk associated with ciprofloxacin versus levofloxacin. Although we observed a trend toward greater risk for MRSA after exposure to levofloxacin than ciprofloxacin, the difference was not significant. Such a difference, if confirmed by further investigation, would be unexpected. On the basis of comparative MIC data, levofloxacin is considered more active than ciprofloxacin against susceptible isolates of *S. aureus*. Our results indicate that levofloxacin may be more likely to promote MRSA and suggest that the effect of levofloxacin in promoting colonization may be stronger than that of ciprofloxacin. Any differences between fluoroquinolones, if proven, would have important implications regarding the clinical decision to choose a particular fluoroquinolone and could shed light on the mechanism of the relationship between these agents and MRSA.

The association between fluoroquinolone exposure and MRSA, established here using rigorous epidemiologic methods, serves as a reminder that the risk factors associated with emerging antimicrobial resistance may not always be predictable or intuitively obvious. Careful consideration must be given to the clinical implications of these findings. In the case of fluoroquinolones and MRSA, decisions promoting the use of a single antimicrobial drug or class of agents could have unforeseen effects on the emergence of antimicrobial resistance.

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Genetic Variation among Temporally and Geographically Distinct West Nile Virus Isolates, United States, 2001, 2002

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Analysis of partial nucleotide sequences of 22 West Nile virus (WNV) isolates collected during the summer and fall of 2001 and 2002 indicated genetic variation among strains circulating in geographically distinct regions of the United States and continued divergence from isolates collected in the northeastern United States during 1999 and 2000. Sequence analysis of a 2,004-nucleotide region showed that 14 isolates shared two nucleotide mutations and one amino acid substitution when they were compared with the prototype WN-NY99 strain, with 10 of these isolates sharing an additional nucleotide mutation. In comparison, isolates collected from coastal regions of southeast Texas shared the following differences from WN-NY99: five nucleotide mutations and one amino acid substitution. The maximum nucleotide divergence of the 22 isolates from WN-NY99 was 0.35% (mean = 0.18%). These results show the geographic clustering of genetically similar WNV isolates and the possible emergence of a dominant variant circulating across much of the United States during 2002.

West Nile virus (WNV) is a member of the genus *Flavivirus* (family *Flaviviridae*) and belongs to the Japanese encephalitis virus serocomplex. Until 1999, the geographic distribution of the virus was limited to Africa, the Middle East, India, and western and central Asia with occasional epidemics in Europe (1,2). By December 2002, however, the distribution of the virus had expanded to include 44 states of the continental United States and southern regions of 5 Canadian provinces from Saskatchewan to Nova Scotia (3). Over the course of 3 years, the virus has traversed North America, presumably from New York City, where it was first isolated during the summer of 1999 (4–7). Partial nucleotide and complete genome sequence analysis of several WNV strains isolated in the northeastern United States during 1999 and 2000 showed that these isolates were most closely related to a WNV strain isolated from the brain of a dead goose in Israel in 1998 (6,8,9). The subsequent establishment of WNV across the eastern and midwestern regions of North America from 1999 through 2001 set the stage for the rapid and widespread movement of the virus across the remainder of the continent during the summer of 2002, resulting in the highest number of annual case reports and deaths attributed to WNV in humans, equines, and birds documented since the discovery of the virus in North America. Surveillance programs initiated by public health agencies, research institutions, and diagnostic laboratories have resulted in the collection of hundreds of WNV isolates across the United States and Canada from various sources, including mosquitoes, humans, equines, birds, and a number of other vertebrate species (3).

Phylogenetic comparisons of partial and complete nucleotide sequences from isolates collected in the northeastern United States during 1999 and 2000 demonstrated a high degree of genetic similarity to the prototype New York strain, WN-NY99 (GenBank accession no. AF196835), with nucleotide identities of $\geq$99.8% and amino acid identities of $\geq$99.9% (9–12). Although these studies have confirmed that northeastern isolates collected in 1999 and 2000 showed limited genetic divergence from WN-NY99, to date little published information has described the continuing divergence of WNV as its temporal and spatial distribution have expanded (13). To assess

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the extent to which WNV has evolved since its introduction in North America, we analyzed the partial nucleotide and deduced amino acid sequences of WNV isolates collected during the summer and fall of 2001 and 2002 and compared them to a homologous sequence region of WN-NY99. Collaborations between the University of Texas Medical Branch (UTMB) and a number of U.S. public health agencies have allowed 22 isolates of WNV to be collected, representing several geographically distinct U.S. regions. Phylogenetic comparisons of a 2,004-nucleotide region encoding the entire premembrane and envelope proteins (prM-E) of each isolate have shown the most divergent variants of WNV in North America to date and provide evidence of the possible emergence of a dominant variant circulating in many regions of the United States. Furthermore, our results indicate geographic clustering of distinct variants within and between states and reinforce previous evidence supporting the likelihood of multiple introductions of virus into the state of Texas (13).

Materials and Methods

Collection and Virus Isolation

Isolates were collected from five states: Illinois, Alabama, Louisiana, Colorado, and Texas. Isolates from Texas were collected from nine counties representing regions across the entire state (Figure 1). All isolates were collected from September 2001 to October 2002. After being confirmed WNV-positive by state public health laboratories, virus or tissues were sent to UTMB for submission into the World Arbovirus Reference Collection. Each sample was given one passage in Vero cells to derive viruses for use in these studies. Virus samples represented a variety of sources, including mosquito pools, bird brain, human cerebrospinal fluid (CSF), and a dog kidney. Of the 18 isolates sequenced in this study (Table 1), 11 were isolated from mosquito pools by the Texas Department of Health (TDH); 2 from a mosquito pool and dog kidney homogenate by the Illinois Natural History Survey (INHS); 2 from passerine brain homogenates from the University of Alabama at Birmingham; 1 from a red-tailed hawk brain homogenate by the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases (CDC-DVBID), Fort Collins, Colorado; 1 from a mosquito pool in Louisiana, courtesy of CDC-DVBID; and 1 from the CSF of a patient who died of West Nile encephalitis at UTMB.

RNA Extraction, Reverse Transcription, and Polymerase Chain Reaction

Viral RNA was extracted directly from 140 µL of infected Vero or BHK cell culture supernatants by using the QiaAMP viral RNA extraction kit (Qiagen, Valencia, CA). Reverse transcription (RT) was performed in a 50-µL volume containing 5 µL of viral RNA, 1 µL of random hexamer primer, 10 µL of 5X RT buffer, 4 µL of 10 mM dNTPs, 0.4 µL of cloned RNAse inhibitor, 0.5 µL of Moloney murine leukemia virus (MMLV) reverse transcriptase, and 29.1 µL of high-performance liquid chromatography (HPLC) water. Polymerase chain reaction (PCR) was performed in a 25-µL volume containing 2.0 µL cDNA template from RT, 1.0 µL forward primer, 1.0 µL reverse primer, 2.5 µL 10X PCR buffer, 0.5 µL 10 mM dNTPs, 0.5 µL of 1 U/µL Taq PCR, and 17.5 µL of HPLC water. Three previously described primer pairs were used to amplify the entire prM-E genes of WNV (13). PCR products were gel-purified by using the QIAquick kit (Qiagen), according to the manufacturer's protocol, and the resulting template was directly sequenced by using the amplifying primers. The WN1751/WN2504A PCR product derived from WNV isolate Galveston County, TX-3 was cloned into pGEM-T Easy (Promega Corporation, Madison, WI), and 10 clones were sequenced to determine the degree of nucleotide sequence divergence within a single isolate collected from the southeast coast of Texas. Sequencing reactions were performed in the UTMB Biomolecular Resource Facility's DNA sequencing laboratory by previously described methods (13). Analysis and assembly of sequencing data were performed by using the Vector NTI Suite software package (Informax, Frederick, MD). Nucleotide and deduced amino acid sequences of the entire prM-E genes from each isolate were aligned by using the AlignX program in the Vector NTI Suite and compared with previously published sequences of isolates from southeast Texas collected from June to August of 2002 (13). All isolates were then compared with isolates collected in the northeastern United States during 1999, 2000, and 2001, and a phylogenetic tree was constructed by maximum parsimony algorithm by using PAUP (Version 4.0b10) (Sinauer Associates, Sunderland, MA) to show genetic relationships of these isolates with other North American WNV isolates found in GenBank, in

Figure 1. Locations of collected isolates, 2001–2002.

Table 1. Nucleotide mutations in sequences of the prM-E genes of 22 West Nile virus isolates obtained during 2001 and 2002 compared to WN-NY99[a]

				prM (501 nt)								
Strain	Source	Collected	RNA origin	491 (prM9)	507	549	621	660	679 (prM72)	690	807	903
WN-NY99 (AF196835)	Flamingo	06/01/99	Brain	A (Lys)	A	U	A	C	U (Ser)	C	C	G
Harris Co., TX (AY185906)	Bluejay	06/11/02	Brain/Vero					U				
Harris Co., TX (AY185907)	Bluejay	06/10/02	Brain/Vero					U				
Nueces Co., TX-1	*Culex quinquefasciatus*	08/06/02	BHK	G				U				
Nueces Co., TX-2	*C. quinquefasciatus*	09/17/02	BHK					U				
Gregg Co., TX	*C. quinquefasciatus*	09/25/02	BHK					U				
Tarrant Co., TX	*C. restuans*	09/30/02	Vero									
Wichita Co., TX	*C. quinquefasciatus*	10/23/02	BHK					U				
Randall Co., TX	*C. tarsalis*	09/18/02	BHK					U				
El Paso Co., TX	*C. tarsalis*	08/26/02	Vero					U				
Illinois-1	*C. pipiens*	08/02/02	Vero			A				U		
Illinois-2	Dog	08/01/02	Kidney/Vero		G			U				
Alabama-1	*C. quinquefasciatus*	10/05/01	Vero									
Alabama-2	Crow	09/10/01	Brain/Vero									
Colorado	Red-tailed Hawk	08/01/02	Brain/Vero					U				
Louisiana	*C. salinarius*	08/06/02	Vero								U	
Galveston Co., TX-1 (AY185914)	Bluejay	08/02/02	Vero				G		A			
Galveston Co., TX-2 (AY185913)	Bluejay	07/19/02	Vero						A			
Galveston Co., TX-3	*C. quinquefasciatus*	08/21/02	Vero									
Jefferson Co., TX-1	*C. quinquefasciatus*	08/06/02	BHK									
Jefferson Co., TX-2	*C. quinquefasciatus*	07/02/02	BHK									A
Jefferson Co., TX-3	Human	08/24/02	CSF/Vero									
Orange Co., TX	*C. quinquefasciatus*	07/03/02	Vero									A

[a]Nucleotide (nt) numbers correspond to those of WN-NY99 (GenBank accession no. AF196835); in brackets for individual residues are the deduced amino acid changes.

which the homologous 2,004-nucleotide region had been sequenced.

Results

Nucleotide sequences representing a 2,004-nucleotide region of the complete prM-E genes of WNV (nucleotides 466–2,469) of the 18 isolates collected in 2001 and 2002 (GenBank AY4281514–AY428531), plus 4 southeast Texas strains (13), were compared with a homologous sequence region of the prototype WNV, WN-NY99 (Tables 1 and 2). Of the 22 isolates analyzed, 16 were collected from 10 different Texas counties, and 2 each from Illinois and Alabama, plus 1 each from Colorado and Louisiana. All isolates were from 2002, except 2 that came from Alabama in 2001 (Figure 1). Sequence alignments comparing WN-NY99 with individual 2001 and 2002 isolates showed up to seven nucleotide mutations and three amino acid substitutions among the 22 isolates analyzed (Tables 1 and 2). Nucleotide mutations occurred at 33 positions (9 in prM, 24 in E) with a total of 7 amino acid substitutions (2 in prM, 5 in E). The maximum nucleotide divergence of the 22 isolates from WN-NY99 was 0.35%, with an average nucleotide divergence of 0.18%.

Several of the nucleotide mutations identified in this study were shared by many isolates (Table 1 and 2; Figure 2). Two nucleotide mutations at residues 1,442 (conservative amino acid substitution of Val to Ala at position E159) and 2,466 were shared by 14 of the 22 isolates, with 10 of these 14 isolates sharing an additional noncoding nucleotide mutation at residue 660. Five different nucleotide mutations (at residues 969, 1,192 [amino acid substitution of Thr to Ala at position E76], 1,356, 2,154, and 2,400) were shared by seven isolates, all of which were collected from coastal regions of southeast Texas. The isolate from Louisiana differed from WN-NY99 at only one nucleotide (residue 807) over the region studied and did not share any nucleotide mutations with other isolates from this study. In comparison, all other nucleotide mutations identified in this study were not shared by nucleotide sequences reported previously from isolates collected in the northeastern United States during 1999, 2000, or 2001 (9–12). Because these mutations were unique to isolates sequenced during this study, our results did not show a closer genetic relationship to isolates from 2001, 2000, or 1999. However, the two isolates in this study that were collected in 2001 (Alabama-1; Alabama-2) did share two nucleotide mutations (residues 1,442 and 2,466) with 12 of the other isolates collected in 2002. Construction of a phylogenetic tree by maximum parsimony analysis (Figure 3) illustrates the genetic proximity of isolates from this study

Table 2. Nucleotide mutations in sequences of the prM-E gene of 22 West Nile virus isolates obtained during 2001 and 2002 compared to WN-NY99

Strain	Envelope (1,503 ntds)																							
	969	1,038	1,065	1,071	1,118 (E51)	1,137	1,179 (E71)	1,192 (E76)	1,293	1,356	1,377	1,442 (E159)	1,443	1,554	1,557	1,581	1,728	1,830	2,094	2,154	2,190	2,392 (E476)	2,400	2,466
WN-NY99 (AF196835)	C	U	C	U	C (Ala)	C	A (Lys)	A (Thr)	C	C	C	U (Val)	U	T	C	T	A	T	A	U	U	G (Ala)	U	C
Harris Co., TX (AY185906)												C										A		U
Harris Co., TX (AY185907)			U			U						C												U
Nueces Co., TX-1											U	C									C			U
Nueces Co., TX-2					U				U		U	C												U
Gregg Co., TX												C	C											U
Tarrant Co., TX		C										C												U
Wichita Co., TX												C												U
Randall Co., TX					U		C					C												U
El Paso Co., TX												C												U
Illinois-1												C												U
Illinois-2												C			U	C		C						U
Alabama-1												C												U
Alabama-2												C					T							U
Colorado												C							G					U
Louisiana																								
Galveston Co., TX-1 (AY185914)	U							G		U										C			C	
Galveston Co., TX-2 (AY185913)	U							G		U										C			C	
Galveston Co., TX-3	U							G		U				C						C			C	
Jefferson Co., TX-1	U							G		U										C			C	
Jefferson Co., TX-2	U			C				G		U										C			C	
Jefferson Co., TX-3	U							G		U										C			C	
Orange Co., TX	U			C				G		U										C			C	

[a]Nucleotide numbers correspond to those of WN-NY99 (GenBank accession no. AF196835); in brackets for individual residues are the deduced amino acid changes.

to those collected from the northeastern United States in 1999, 2000, and 2001. Branch groupings showed both temporal and geographic separation of isolates, with those collected in the northeastern United States in 1999, 2000, and 2001 representing a distinct clade in relation to isolates collected in 2002. An exception to this grouping is an isolate from Louisiana collected in 2002, which was grouped with northeastern United States isolates from 1999 to 2001. Notably, WNV isolates from the southeastern coast of Texas also comprise a clade of their own, separating these isolates from other 2001 and 2002 isolates collected from various regions within the United States. A recently reported WNV isolate collected from a Missouri dog in 2002 (GenBank accession no. AY160126) also shared a nucleotide mutation (residue 2,466 C to U) with the 2002 isolates from this study. Although the entire prM-E gene of this isolate was not reported, this isolate likely represents an additional member of the large 2002 clade.

In a previous report concerning the genetic divergence of WNV since its introduction into the United States, Beasley et al. (13) described a quasispecies population within a single WNV isolate from Harris County, Texas. To determine whether nucleotide mutations that define the southeast coastal Texas variant were uniform throughout the quasispecies population of a select isolate, the WN1751/WN2504A PCR product derived from WNV isolate Galveston Co., TX-3, was cloned into pGEM-T Easy. Ten clones were sequenced to obtain homologous regions of 700 nucleotides, which were then compared with the Galveston Co., TX-3, consensus sequence. This region contained the U to C mutation at nucleotide 2154 and the U to C mutation at nucleotide 2,400. Five of the 10 clones were identical to the consensus sequence, while the other five clones each had one or two nucleotide changes from the consensus sequence for a total of eight nucleotide changes (Table 3). None of the mutations identified represented amino acid substitutions and, unlike the 2001–2002 variant population (13), none of the mutations encoded a stop codon. The maximum nucleotide divergence of individual clones was 0.28% (mean = 0.11%). Furthermore,

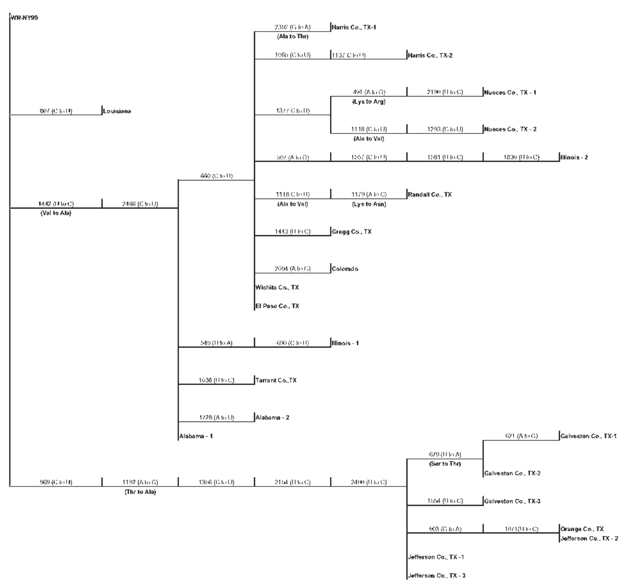

Figure 2. Phylogram based on maximum parsimony analysis comparing a 2,004-nucleotide sequence of WN-NY99 (Gen Bank accession no. AF196835) with 22 West Nile virus isolates collected during 2001 and 2002.

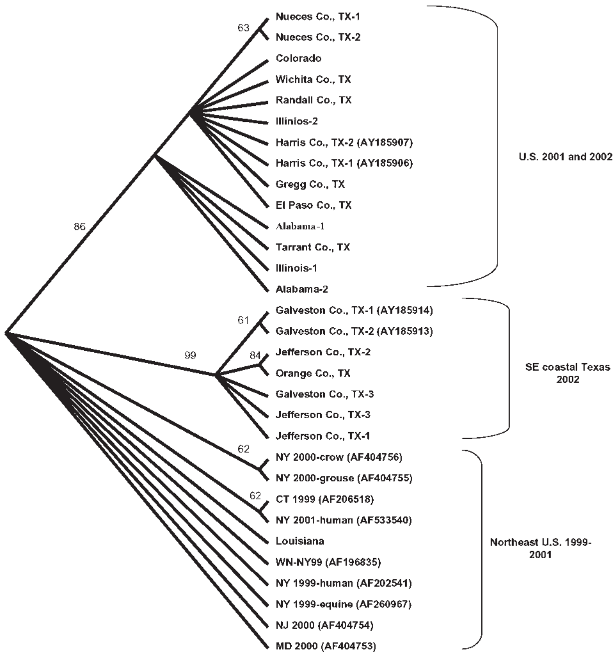

Figure 3. Cladogram based on maximum parsimony analysis comparing a 2,004-nucleotide sequence of 22 West Nile virus isolates collected during 2001 and 2002 with a homologous region of WN virus isolates collected in 1999, 2000, and 2001 from the northeastern United States. Numbers indicate bootstrap confidence estimates based on 500 replicates for clades supported to the right. Numbers in parenthesis represent GenBank accession numbers.

none of the nucleotide changes identified in the five clones was shared with WNV strains representing the 2001–2002 variant, nor were any nucleotide changes identified at two of the nucleotide positions that defined the southeastern coastal Texas variant. These results suggest that none of the virus genomes existing in a quasispecies population from WNV isolate Galveston Co., TX-3, contained nucleotide mutations characteristic of the 2001–2002 variant identified in this study.

Discussion

Sequence comparisons of a 2,004-nucleotide region of 22 WNV isolates collected during the summer and fall of 2001 and 2002 showed the highest degree of nucleotide divergence from WN-NY99 to date. Studies by Lanciotti et al. (9) and Huang et al. (12) have shown that the complete genomes of several WNV isolates collected in 1999, 2000, and 2001 share $\geq$99.8% nucleotide identity with WN-NY99, with three or fewer amino acid substitutions in the entire polyprotein. Similar studies of partial nucleotide sequences conducted by Anderson et al. (10) and Ebel et al. (11) reported up to three nucleotide mutations encompassing a region of 921 nucleotides and 1,503 nucleotides from isolates collected in Connecticut in 1999 and 2000 and New York in 2000, respectively. Although our studies have compared a larger portion of the genome than earlier studies of partial nucleotide sequences, we have identified individual isolates with as many as seven nucleotide mutations and three amino acid substitutions, with a maximum divergence of 0.35% from the homologous region of the prototype North American WNV, WN-NY99. The nucleotide mutations identified in this study were not shared by previously sequenced isolates from 1999, 2000, or 2001 (9–12) and represent new nucleotide changes in the North American WNV population. Since these changes were not shared with other previously reported WNV sequences, the isolates analyzed in this study did not show a greater genetic similarity with northeastern isolates from 1999, 2000, or 2001. However, several of these nucleotide changes (660, 969, 1,356, 2,154, 2,400, and 2,466) are observed in other Old World WNV strains from both lineage I and lineage II (Table 4). Each of these changes represents a noncoding mutation from either a C to U or U to C in the third codon of the open reading frame; nucleotides at these positions may revert back to nucleotides observed in the more ancestral Old World strains.

Our results also suggest the geographic clustering of genetically distinct variants. Seven of the 22 isolates, all of which were collected from coastal regions of southeast Texas, share five nucleotide mutations unique to only these isolates. Fourteen of the other isolates, which represent the CDC-defined East South Central (AL), West South Central (LA and TX), East North Central (IL), and Mountain (CO) regions (3), all share two unique nucleotide mutations not identified in other isolates (Figure 2). The results of this

Table 3. Nucleotides that varied among individual clone sequences of a fragment of the E protein gene (genomic positions 1,769-2,469) of the WNV Galveston Co., TX-3[a,b]

Clone	Nucleotide							
	1,779	1,787	1,798	1,871	2,162	2,168	2,232	2,469
Consensus sequence	U	U	A	A	A	G	A	U
1			G				G	
2	C	C						
4				G				C
6					G			
7						A		

[a]WNV, West Nile virus.
[b]Clones 3, 5, 8, 9, 10 are identical to the consensus sequence.

Table 4. Nucleotide changes from WN-NY99 observed in 2001 and 2002 WNV isolates that are conserved in Old World WNV isolates with complete genomes available from GenBank[a,b]

660 (C to U)	969 (C to U)	1,356 (C to U)	2,154 (U to C)	2,400 (U to C)	2,466 (C to U)
WN Uganda 1937 (M12294) WN LEIV-Krnd88-190 (AY277251)	WN IS-98 STD (AF481864) WN Eg101 (AF260968) WN Ast99-901 (AY278441) WN LEIV-Krnd88-190	WN Eg101	WN Uganda 1937	WN Uganda 1937 WN LEIV-Krnd88-190 WN Eg101 WN Ast99-901 WN RO97-50 (AF260969) WN VLG-4 (AF317203) WN KN3829 (AY262283) WN Italy 1998-equine (AF404757) WN LEIV-Vlg00-27924 (AY278442) WN VLG-4 (AF317203)	WN Uganda 1937

[a]WNV, West Nile virus.
[b]Number in parenthesis represents GenBank accession no.

study support the findings of Beasley et al. (13), which suggest that during the summer of 2002 WNV was introduced into Texas on at least two separate occasions. These results might reflect the unique migratory patterns of North American birds, which act as reservoir hosts for WNV. As Rappole et al. (14) have illustrated, many North American birds follow well-documented migration routes from summer grounds in the northeastern United States to southern areas that are classified as the southeastern United States, circum-Gulf, trans-Gulf, and Caribbean/western North Atlantic routes. For example, the Laughing Gull (*Larus atricilla*) has been known to follow a circum-Gulf route as it travels from the northeastern United States to stopover sites along the northern and western Gulf Coast on its way to Mexico or Central America. Because certain species of birds have a more limited geographic range than others, geographically clustered populations of distinct genetic variants, for example, isolates collected from coastal regions of southeast Texas, might arise as a result of restricted migratory routes. This hypothesis is supported by a number of studies. Peiris and Amerasinghe (15) have identified a group of geographically restricted antigenic variants of WNV confined to southern India. Because of the lack of bird migratory routes linking southern India with the Middle East and Africa, a distinct antigenic group exists exclusively in southern India. Furthermore, numerous studies have shown antigenic variation among WNV strains that correlate with geographically distinct regions and restricted bird migratory patterns (16,17). Phylogenetic comparisons of Indian viruses with other WNV strains show similar findings, which place Indian WNV strains in a unique clade of lineage I (9,18). Recent studies in Israel by Malkinson et al. (19) also support the role of migratory birds in the dispersion of unique WNV variants in geographically distinct regions. The results of our study support an alternative hypothesis that explains the continental spread of WNV as a consequence of transmission between local bird and mosquito populations in a given region. This mechanism allows for spread of the virus from region to region over shorter distances, in contrast to the long distances traveled by migratory birds (20). Our finding of a dominant variant that exists over a large part of the United States, together with evidence of a geographically distinct southeast coastal Texas variant, suggests that both mechanisms of spread have influenced the genetic distribution and spread of WNV in the United States.

To date, little genetic evidence supports or refutes the hypothesis that WNV becomes established in an enzootic transmission cycle in a particular geographic area rather than being reintroduced into a particular area each year when the transmission season begins. Similarly, because of the limited published data detailing the year-to-year genetic changes observed in WNV, whether the virus is becoming endemic in particular regions of the United States remains to be established. This question will be answered in part by determining baseline phylogenetic results of specific variants in a geographic area and by analyzing isolates collected in sequential transmission seasons.

Although the isolates analyzed in this study do not represent the entire temporal and geographic distribution of WNV in North America, at least some nucleotide mutations have been conserved among WNV strains circulating across the continent. If indeed the conservation of these mutations is the result of selective pressure, such as the continued capacity to replicate in both arthropod and vertebrate hosts, rather than random mutations occurring as a consequence of genetic drift, one would expect these mutations to be conserved in virus isolates collected in other regions of North America. Further investigation concerning the genetic composition of viruses from additional regions of North America will define the extent to which dominant variants have emerged. If dominant variants do continue to emerge across the United States, phylogenetic analyses will help researchers monitor the spread of WNV in North America and may provide explanations for the rapid and widespread movement of this newly emerging virus in North America. Similarly, identifying the genetic composition of WNV isolates from other regions of the United States and Canada, as well as comparing these isolates with isolates collected in 2003, will continue to define evolutionary relationships of WNV circulating in North America and facilitate predictions concerning the primary mechanisms of transmission and spread of the virus.

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Ebola Hemorrhagic Fever Transmission and Risk Factors of Contacts, Uganda[1]

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From August 2000 through January 2001, a large epidemic of Ebola hemorrhagic fever occurred in Uganda, with 425 cases and 224 deaths. Starting from three laboratory-confirmed cases, we traced the chains of transmission for three generations, until we reached the primary case-patients (i.e., persons with an unidentified source of infection). We then prospectively identified the other contacts in whom the disease had developed. To identify the risk factors associated with transmission, we interviewed both healthy and ill contacts (or their proxies) who had been reported by the case-patients (or their proxies) and who met the criteria set for contact tracing during surveillance. The patterns of exposure of 24 case-patients and 65 healthy contacts were defined, and crude and adjusted prevalence proportion ratios (PPR) were estimated for different types of exposure. Contact with the patient's body fluids (PPR = 4.61%, 95% confidence interval 1.73 to 12.29) was the strongest risk factor, although transmission through fomites also seems possible.

Ebola hemorrhagic fever (EHF) is a severe viral disease caused by three of the four species of "Ebola-like viruses" (1), which are probably maintained in an as-yet-undefined natural reservoir in the rain forests of Africa (2). Epidemics occur when an infectious case-patient is introduced into a susceptible population. The first recognized epidemics occurred almost simultaneously in 1976 in southern Sudan (284 cases and 117 deaths) (3) and in a nearby region of the Democratic Republic of Congo (318 cases and 280 deaths) (4). A major mode of transmission was within hospitals, especially in the early stages of the outbreaks. Person-to-person transmission also occurred outside the hospital setting, with numerous community-acquired cases (3,4).

In 1995, another large epidemic occurred in Kikwit, in the Democratic Republic of Congo, with 315 cases and 244 deaths (5). The primary mode of transmission was person-to-person transmission to household members who had had direct contact with sick persons or their body fluids, especially during the late stage of the disease (6). However, the source of infection remained unknown for 12 case-patients, which led to the suspicion that the virus was transmitted by airborne particles or fomites (7).

The largest epidemic (425 presumptive cases and 224 deaths) occurred from August 30, 2000 (i.e., the earliest presumptive case), to January 9, 2001 (i.e., onset of the last case), in the Republic of Uganda, which borders both the Democratic Republic of Congo and Sudan (8–11). Since then, epidemics have been occurring with increasing frequency. Specifically, between December 2001 and March 2002, outbreaks occurred in the Republic of Gabon (65 cases and 53 deaths) (12,13) and in the neighboring Republic of Congo (57 cases and 43 deaths) (12). In February 2003, cases again began to be reported in the Republic of Congo, where 13 laboratory-confirmed case-patients and 127 epidemiologically linked case-patients, including 123 deaths, have been reported to date (14).

During the epidemic in Uganda, a national task force, in collaboration with an international team of health professionals, conducted activities for controlling the epidemic and managing cases (11). The area in which the epidemic was mainly concentrated was the Gulu District, a savannah area located in the north and mainly inhabited by Nilotic

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[1]This paper is dedicated to Dr. Matthew Lukwiya, Medical Superintendent of St. Mary's Hospital Lacor, and the other health staff who contracted and died of Ebola while taking care of hospital patients.

tribes. Most of the district's 400,000 inhabitants live in Gulu Town or in one of several camps, located in rural areas, for persons who have been internally displaced because of the insecurity caused by the activity of insurgents.

On October 8, an outbreak of EHF was suspected at St Mary's Hospital Lacor (hereafter termed Lacor Hospital), a nonprofit facility located several kilometers from Gulu Town. Two days later, isolation wards were set up in the district's major hospitals, i.e., Lacor Hospital and the Gulu Government Hospital. In Lacor Hospital, only the hospital staff provided patient care in the isolation ward, whereas in the Gulu Government Hospital, relatives were allowed to contribute, which is the usual practice in Ugandan hospitals. The staff of both hospitals adopted strict barrier nursing precautions (e.g., gloves, masks, gowns, aprons, rubber boots); in the Gulu Government Hospital, these precautions were partially extended to patients' relatives. On October 15, the outbreak was confirmed, and a system of daily case reporting, including a computerized database, was established. A case-patient was defined as a person who experienced at least one of the following events (9,10): 1) unexplained bleeding; 2) abrupt onset of fever and three or more of the following symptoms or signs: headache, vomiting, anorexia, diarrhea, weakness, or severe fatigue, abdominal pain, body aches or joint pain, difficulty in swallowing, difficulty in breathing, and hiccups; and 3) death from unexplained causes.

On October 21, 2000, the Centers for Disease Control and Prevention (CDC) set up a laboratory for performing enzyme-linked immunosorbent assays (ELISAs) for Ebola antigens and antibodies and reverse transcriptase-polymerase chain reaction (RT-PCR) at Lacor Hospital. Laboratory confirmation (positive result for Ebola virus antigen or Ebola immunoglobulin [Ig] G antibody) was obtained for 218 (51.3%) of the total 425 presumptive cases involved in the epidemic (9,10).

At approximately the same time, a surveillance system for contact tracing and case finding was established. A contact was defined as a person who had at least one of the following exposures: 1) physical contact with a case-patient, alive or dead; 2) slept in the same hut or house with a case-patient during the disease period; 3) contact with a case-patient's body fluids during the disease period; and 4) contact with a case-patient's linens or other possible fomites during the disease period and just after death. Members of the surveillance teams and the hospital staff were not considered contacts, even if they were exposed to a case-patient, because they had been taught how to protect themselves. For each case-patient, a list of contacts was created; all contacts were followed by daily home visits for 21 days (maximum incubation period) from the last contact with the case-patient (11).

In November and December 2000, we collected additional data from a group of contacts (or their proxies) concerning the nature and timing of their exposure to case-patients. Our objective was to trace chains of transmission and identify risk factors for transmission among a group of exposed persons in the community. This study, the results of which are reported here, was fully integrated into the surveillance activities described above and was authorized by the director of the Gulu District Health Services and the Ugandan Ministry of Health.

Methods

Study Design and Population

To retrospectively trace the chain of transmission, we interviewed three laboratory-confirmed case-patients in the Lacor Hospital who had onset of symptoms October 23–28 (referred to as "study case-patients"; see Table 1 for other definitions). We asked them to identify the persons from whom they had probably acquired the disease (referred to as index patients). In turn, the index patients (or their next of kin living in the same village if they had died, as was usually the case) were then asked to identify the persons from whom they had probably acquired the disease (also referred to as index patients). This process was repeated until we reached the patients whose source of infection could no longer be identified (referred to as primary case-patients). For each of the index patients and primary case-patients, we then reviewed the list of persons

Table 1. Definitions used in the chain of transmission of Ebola hemorrhagic fever

Classification	Definition
Study patients	The three laboratory-confirmed case-patients from whom we retrospectively identified the other case-patients and their contacts
Index patients	The nine case-patients retrospectively identified from the study patients as the source of infection (including primary case-patients)
Primary case-patients	The three earliest patients for whom we were not able to identify the source of infection
Collateral case-patients	Cases generated by the index patients, or by other collateral case-patients, and identified by matching the list of contacts of these persons with the list of reported cases
Postprimary case-patients	Case-patients for whom we were able to identify the source of infection
Contacts	Persons exposed to a case-patient, listed by the surveillance teams using the definition reported in the background section.
Healthy contacts	Contacts in whom the disease did not develop within 21 days of the last exposure

with whom they had been in contact since the onset of their symptoms; such information had been routinely collected as part of surveillance. Then, as 21 days had passed since the last exposure, each name on the list was matched with a name on the list of reported case-patients in order to identify the contacts in whom the disease had developed (collateral case-patients). The process was then repeated prospectively with the collateral case-patients for as many generations as possible.

To identify risk factors, we interviewed all of the identified contacts (or their proxies) of the primary, index, and collateral case-patients, irrespective of their status (patient or healthy contact). To this end, we developed a questionnaire that focused on the exact type and timing of exposure to index patients.

Data Analysis and Statistical Methods

We performed univariate analyses to evaluate the strength of associations between the different types of exposure and disease, by comparing disease prevalence among persons with a given exposure to that among persons without that exposure and by testing the resulting differences with the chi-square test or, when appropriate, the Fisher exact test. Those risk factors independently associated with the disease were evaluated in multivariate analyses by using log-binomial regression models after we ascertained the absence of a significant multiple colinearity among the variables. The crude and adjusted prevalence proportion ratios (PPR) and their 95% confidence intervals (CI) were used to describe the strength of the associations (15).

Results

Chains of Transmission

The Figure illustrates the three reconstructed chains of transmission; each consisted of three identified generations of cases (excluding the study case-patients). The 27 identified case-patients consisted of, in addition to the 3 laboratory-confirmed patients with whom we began the study, 9 index case-patients (including 3 primary case-patients, all young women whose source of infection was unknown), and 15 collateral case-patients. Of the 24 postprimary patients, 14 (58.3%) lived in the Gulu Town or Municipality, and 10 (41.7%) lived in rural areas of the Gulu District. One patient was a newborn, and three were infants. The remaining 20 patients (83.3%) ranged in age from 14 to 70 years; 14 (70.0%) of these 20 patients were female, and most were housewives or subsistence farmers (70.0%).

The 24 postprimary patients had onset of symptoms from September 18 to October 28, 2000. The incubation period (i.e., time elapsed between either the last or the first contact with the index patient and the onset of symptoms) was 1–16 days (median 6 days), when the last contact was considered, and 1–12 days (median 12 days), when the first contact was considered. All three infants had an incubation period of <7 days.

Twenty (83.3%) of the 24 postprimary case-patients were admitted to the hospital;13 (65.0%) were admitted after the isolation ward had been created. The four patients not admitted to the hospital (a newborn, two infants, and an elderly woman) died within 3 to 11 days of disease onset. Of the 20 hospital patients, 7 were still in the hospital when the laboratory was set up, and 3 were admitted afterwards; all 10 of these patients tested positive for Ebola antigens, IgG, or both.

Of the 20 hospitalized patients, 15 died. Among these 15 patients, the duration of illness (from onset of symptoms to death) was 3–15 days (median 10 days); the duration of hospitalization (from admission to death) was 2–11 days (median 5 days). Among the five surviving patients, the duration of illness (from onset of symptoms to discharge upon clinical recovery) was 10–25 days (median 15 days); the duration of hospitalization was 8–22 days (median 13 days).

Of the 27 patients, all of the primary and secondary case-patients died. Of the remaining 17 patients, 12 (70.6%) died. Of the four persons who died without being admitted to the hospital, two had secondary cases and two had tertiary cases.

In the legend to the Figure, the 27 cases are briefly described and the mode of transmission is summarized for the 24 postprimary cases. The newborn (case-patient 20) was delivered by a sick woman 4 days after the onset of symptoms, and the other three infants (case-patients 2, 9, and 26) had been breastfed by sick mothers. The other 20 postprimary cases were all members of the extended family (household contacts) of the case-patients to whom they had been exposed. All but one (95%) had had direct physical contact with the patient who was the likely source of their disease; the remaining person (case-patient 7) had slept wrapped up in a blanket left by his brother, who had just died of EHF.

Among the 20 postprimary case-patients who were ≥14 years of age, 15 (75.0%) reported that they had been exposed to the body fluids of their index patient; 11 (55.0%) had washed the index patient's clothes; and 18 (90.0%) had taken care of the index patient at some point during his or her illness. Twelve of these 18 persons had taken care of the index patient until death, either in the hospital (n = 6) or at home (n = 6). Eleven (55.0%) of these 20 postprimary patients had slept in the same hut or house as the index patient; of these, 5 had slept with the index patient on the same mat or mattress. Six (30.0%) of these 20 postprimary patients had shared meals with index patients (picking up food with their fingers from the same

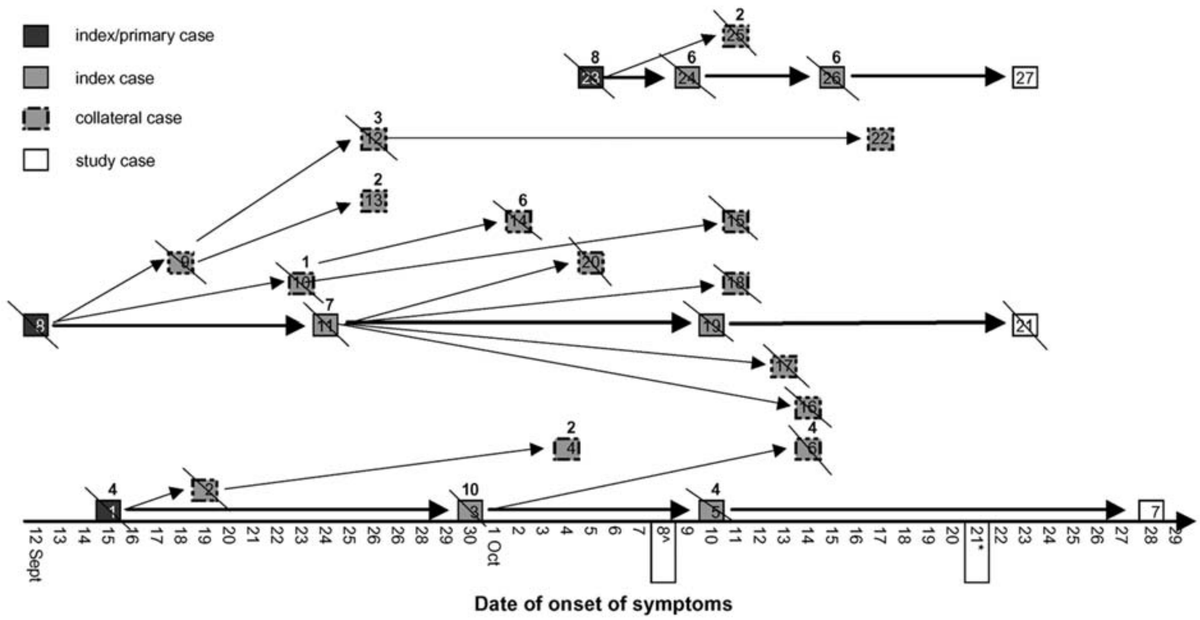

Figure. Chains of transmission relative to 27 Ebola cases, Gulu District, Uganda (September–October 2000). The numbers above the blocks indicate the total number of healthy contacts identified for that patient. The slashes indicate patients who died. The isolation ward was opened on October 8. *A laboratory facility for serologic diagnosis of Ebola was set up at Lacor Hospital by the Centers for Disease Control and Prevention on October 21. Description of individual cases follows: 1. AF, young woman, admitted at Gulu Hospital Sept. 19, died the same day. She was buried the following day, next to her parents' house, without known identified case-patients among those who attended the burial ceremony; 2. OS, son of AF (4 months old); breastfed even during the last days of his mother's life; admitted to Lacor Hospital; died Sept. 30. 3. OA, father-in-law of AF; nursed his daughter-in-law during the last days of the disease, both at home and in the hospital; reported contact with blood and vomit; died Oct. 7. 4. AR, grandmother of OS; nursed the child after the mother's death; reported contact with feces and urine; survived. 5. ON, cousin of OA, whom ON touched before and after OA's death; no reported contacts with body fluid; died Oct. 16. 6. OR, brother of OA; nursed him during last days; reported contacts with feces; died Oct. 17. 7. OJ, brother of ON; used the blanket left by his brother; survived. 8. AE, young woman who sold beer to soldiers; died Sept. 17. 9. OS, son of AE; breastfed; died Sept. 21. 10. AD, mother of AE; nursed her; reported contact with vomit and feces; prepared the dead body; died Oct. 1. 11. AJ, sister of AE; nursed her; reported contact with feces and vomit; prepared the dead body; died Oct. 4. 12. AV, aunt of OS; nursed the child after the mother's death; reported contact with vomit and feces; died Oct. 11. 13. AN, cousin of OS; they slept together; reported contact with vomit and feces; survived. 14. AV, daughter of AD; nursed her; reported contact with vomit and feces; died Oct. 7. 15. AV, niece of AD; reported direct contact with her during illness; died Oct. 23. 16. AS, daughter of AJ; nursed her, both at home and in the hospital; no reported contact with body fluids; died Oct. 24. 17. AS, co-wife of A J; nursed her; reported contact with blood; died Oct. 24. 18. AE, sister-in-law of AJ; assisted her during delivery on Sept. 28; died Oct. 17. 19. LV, aunt of AJ; assisted her during delivery on Sept. 28; died Oct. 22. 20. OW, son of AJ; born on Sept. 28; died Oct. 9. 21. OJ, husband of LV; nursed her; died Nov. 1. 22. AC, cousin of AV; nursed her; reported contact with feces; survived. 23. AG, young woman; lived next to the barracks; died Oct. 8. 24. AL, sister of AG; nursed her; reported contact with vomit; died Oct. 18. 25. JB, sister of AG; nursed her; reported contact with vomit; died Oct. 26. OR, son of AL; breastfed; died Oct. 21. 27. AF, grandmother of OR; nursed the child after the mother's death; reported contact with feces and vomit; survived.

plate). Sixteen (80.0%) of the 20 adult postprimary patients had attended the funeral of their index patient; 11 had also prepared the body for the ceremony or simply touched the dead body; 11 had participated in the communal meal during the ceremony; and 7 had participated in the ritual handwashing during the ceremony.

Healthy Contacts

We also interviewed the 65 apparently healthy contacts of the 9 index patients and 15 collateral case-patients. Notably, not all patients generated contacts, and the six who did not were all third- (n = 5) or fourth-generation case-patients. Five had had onset of symptoms after the isolation ward was created.

Of the 65 healthy contacts, 39 (60.0%) lived in the Gulu Municipality and 23 (35.4%) in rural areas of the Gulu District; information on residence was not available for the remaining three. Two of the healthy contacts (3.1%) were infants, and four (6.2%) were 3–8 years of age The remaining 59 (90.8%) ranged in age from 10 to 70 years; 33 (55.9%) were female; most were housewives or subsistence farmers (60.0%).

One of the two infants had been separated from his sick mother early in the course of the mother's illness; the other infant had been breastfed during his mother's illness. All four of the children 3–8 years of age had slept in the same hut as their sick parent and had had direct physical contact with their sick parent or relative (none of them had taken care of the sick person). None of these four children was reported to have been in contact with the patient's body fluids.

Of the 59 healthy contacts ≥10 years of age, 50 (84.7%) were extended family members of the patient (household contacts); 9 were neighbors of the patient. Forty-seven (79.7%) had had direct physical contact with the case-patient; 15 (25.4%) had been exposed to body fluids; 18 (30.5%) had washed the patient's clothes; and 25 (42.4%) had taken care of the sick person. Of these 25 persons, 11 had taken care of their relative up to the last days of life, either in the hospital (n = 8) or at home (n = 3). Moreover, 13 (22.0%) had slept in the same hut as the patient; 4 had shared the same mat; 7 (11.9%) had shared meals with the index patient (picking up food with their fingers from the same plate).

Thirty-seven (62.7%) of these 59 healthy contacts had attended the funeral of the patient; 14 of them had also touched the dead body. In addition, 14 healthy contacts had participated in the communal meal during the ceremony, and 9 had participated in the ritual handwashing.

Risk Factors

Because of their particular exposures, infants ≤2 years were excluded from the analysis of risk factors. Among the 83 remaining contacts, disease developed in 20. Sixty-three contacts remained healthy. Among contacts, neither age (>30 years vs. ?30 years: PPR = 1.38, 95% CI 0.64 to 2.97) nor sex (women vs. men: PPR = 1.54, 95% CI 0.66 to 3.60) was significantly associated with the disease (Table 2).

Contact with body fluids showed a strong association (PPR = 5.30, 95% CI 2.14 to 13.14). Persons who had had direct physical contact with a sick person were more likely to have acquired the disease (PPR = 3.53, 95% CI 0.52 to 24.11), as were those who had touched the body

Table 2. Univariate analysis of risk factors for Ebola hemorrhagic fever among a population of 83 contacts, Gulu, Uganda, 2000

Risk factors	No. of cases (%)	Crude PPR (95% CI)[a]	p value
Demographic characteristics			
Sex			
Male	6 (18.2)	1	
Female	14 (28.0)	1.54 (0.66 to 3.60)	0.446
Age group (y)			
≤30	9 (20.5)	1	
>30	11 (28.2)	1.38 (0.64 to 2.97)	0.571
Direct transmission			
Touched sick person			
No	1 (7.7)	1	
Yes	19 (27.1)	3.53 (0.52 to 24.11)	0.173
Touched body of deceased person			
No	9 (17.6)	1	
Yes	11 (34.4)	1.95 (0.91 to 4.17)	0.141
Contact with body fluids			
No	5 (9.4)	1	
Yes	15 (50.0)	5.30 (2.14 to 13.14)	<0.001
Indirect transmission			
Shared meals			
No	14 (20.6)	1	
Yes	6 (40.0)	1.94 (0.89 to 4.22)	0.178
Washed clothes			
No	9 (18.8)	1	
Yes	11 (31.4)	1.68 (0.78 to 3.60)	0.283
Slept in the same hut/on the same mat			
No	9 (16.4)	1	
Shared only the hut	6 (35.3)	2.16 (0.90 to 5.19)	
Shared also the same mat	5 (45.5)	2.78 (1.15 to 6.70)	0.019 (for trend)
Ritual handwashing during funeral			
No	13 (19.4)	1	
Yes	7 (43.7)	2.25 (1.08 to 4.72)	0.054
Communal meal during funeral			
No	9 (15.5)	1	
Yes	11 (44.0)	2.84 (1.35 to 5.98)	0.012

[a]PPR, prevalence proportion ratios; CI, confidence interval.

Table 3. Univariate analysis of risk factors for Ebola hemorrhagic fever related to patient care and the number of types of direct contact among 83 contacts, Gulu, Uganda, 2000

Risk factors	No. cases (%)	Crude PPR (95% CI)[a]	p value
Cared for patient			
No	2 (5.0)	1	
Cared only during patient's early stage	6 (30.0)	6.00 (1.33 to 27.10)	
Cared until the patient's death at hospital the hospital	6 (42.9)	8.57 (1.95 to 37.66)	
Cared until the patient's death at home	6 (66.7)	13.33 (3.20 to 55.59)	<0.001 (for trend)
Number of types of direct contact			
No direct contact	1 (16.7)	1	
One type of direct contact	1 (2.9)	0.18 (0.01 to 2.45)	
Two types of direct contact	10 (32.3)	1.94 (0.30 to 12.44)	
Three types of direct contact	8 (66.7)	4.00 (0.64 to 25.02)	<0.001 (for trend)

[a]PPR, prevalence proportion ratios; CI, confidence interval.

of the deceased person (PPR = 1.95, 95% CI 0.91 to 4.17), although these associations were not statistically significant.

Regarding indirect transmission, sleeping on the same mat (PPR = 2.78, 95% CI 1.15 to 6.70), participating in the ritual handwashing during the funeral ceremony (PPR = 2.25, 95% CI 1.08 to 4.72), and sharing a communal meal during the funeral ceremony (PPR = 2.84, 95% CI 1.35 to 5.98) were significantly associated with disease. Although the differences were not statistically significant, sharing meals, washing clothes, and sleeping in the same hut were associated with a higher risk of acquiring the disease.

In general, having taken care of a sick person represented a strong risk factor, although the level of risk was lower for persons who had provided care only at the early stage of the disease (PPR = 6.00, 95% CI 1.33 to 27.10), followed by the risk for those who provided care until the index patient's death, either at the hospital (PPR = 8.57, 95% CI 1.95 to 37.66) or at home (PPR = 13.33, 95% CI 3.20 to 55.59) (Table 3).

The risk tended to increase with the increasing number of different types of direct contact (chi square for trend $p < 0.001$); the risk was higher among persons who were exposed through two (PPR = 1.94, 95% CI 0.30 to 12.94) or three different types of direct contact (PPR = 4.00, 95% CI 0.64 to 25.02), compared with the risk for those who had no direct contact (Table 3).

Factors related to direct and indirect transmission were analyzed separately in multivariate analyses (Table 4). The first model (i.e., factors related to direct transmission) showed that having had contact only with body fluids (adjusted PPR = 4.61, 95% CI 1.73 to 12.29) was strongly associated with the disease, whereas having only touched the patient during illness was not (adjusted PPR = 1.56, 95% CI 0.19 to 13.04). (The weak association found in the univariate analysis was probably confounded by contact with the patient's body fluids.) Having touched the body of the deceased person (adjusted PPR = 1.84, 95% CI 0.95 to 3.55) showed a borderline significant association.

The second model (i.e., factors related to indirect transmission and controlled for the potential confounding effect attributed to the number of different types of direct contact) showed that sleeping in the same hut (adjusted PPR =

Table 4. Multivariate analyses on risk factors for Ebola hemorrhagic fever related to direct and indirect transmission among 83 contacts, Gulu, Uganda, 2000

Risk factors	Adjusted PPR[a]	95% CI[b]	p value
Model 1: Direct transmission			
Touching patient during illness	1.56	0.19 to 13.04	0.679
Touching dead body	1.84	0.95 to 3.55	0.069
Contact with patient fluid	4.61	1.73 to 12.29	0.002
Model 2: Indirect transmission[c]			
Sharing meals	1.69	1.00 to 2.85	0.050
Washing clothes	1.02	0.47 to 2.22	0.957
Sleeping in the same hut/on the same mat			
Sharing only the hut	2.34	1.13 to 4.84	0.022
Sharing also the mat	2.93	1.16 to 7.38	0.023
Ritual handwashing during funeral	1.16	0.54 to 2.49	0.706
Communal meal during funeral	1.50	0.98 to 2.28	0.060

[a]PPRs, prevalence proportion ratios adjusted for all the variables included in the model.
[b]CI, confidence intervals.
[c]Model 2 has been run controlling for the potential confounding effect due to the intensity of direct contacts with a case-patient (less than two types of direct contacts versus two or more types of direct contacts).

2.34, 95% CI 1.13 to 4.84) and sleeping on the same mat (adjusted PPR = 2.93, 95% CI 1.16 to 7.38) were independent risk factors. However, weak associations were found for sharing meals with a sick person and participating in the communal meal during the funeral, whereas the ritual handwashing during the funeral and washing the sick person's clothes were not risk factors.

Discussion

Although the number of EHF epidemics in sub-Saharan Africa has been increasing and EHF viruses have recently been classified as agents that could be used as possible biological weapons (16), epidemiologic data on the modalities of transmission are still limited (6) because of the sporadic and sudden nature of outbreaks. In the Ugandan outbreak, the hospital isolation wards have been important in managing cases. This fact was demonstrated by the finding that the patients with onset of symptoms after the institution of these wards on October 10 were the only ones who did not generate contacts, with the exception of an infant born on September 28, who had onset of symptoms on October 5 and died on October 9. Moreover, the higher death rate observed among primary and secondary case-patients (100%), in contrast with that among the most recent case-patients (70.6%), could be explained by the treatment provided in the hospital, though this treatment was mainly supportive.

The reconstruction of the chains of transmission was straightforward for three generations of case-patients, which suggests that person-to-person transmission occurred. Nevertheless, the source of infection of the primary patients remained unknown, although transmission was occurring in the community. As described in the Figure, most of the links in the chain of transmission were deceased; for this reason, most interviews were administered to proxies. Thus, the possibility that a nonhuman natural reservoir may have been involved could not be excluded.

Among the postprimary case-patients, the most important risk factor was direct repeated contact with a sick person's body fluids, as occurs during the provision of care. As expected, the risk was higher when the exposure took place during the late stage of the disease at home. The risk was reduced when the patient stayed in a hospitals, probably because of the use of gloves, even before strict barrier nursing was implemented (6,7).

By contrast, simple physical contact with a sick person appears to be neither necessary nor sufficient for contracting EHF. In fact, one person in whom the disease developed was probably infected by contact with heavily contaminated fomites (patient 7), and many persons who had had a simple physical contact with a sick person did not become infected.

Transmission through contaminated fomites is apparently possible. In fact, the association found for having slept on the same mat or having shared meals with a sick person or with funeral participants remained after controlling for direct contact. However, having washed the clothes of a sick person and having participated in the ritual handwashing during the funeral ceremony were not significant risk factors.

Finally, although we cannot exclude the possibility of airborne transmission, this mode probably plays a minor role, if any. In fact, the association between having slept in the same hut and acquiring the disease was weak and could have been produced by some unidentified confounding variables. Furthermore, the reported Ebola virus aerosol transmission among nonhuman primates (17,18) has been demonstrated in laboratory experiments, which may be irrelevant in the natural context.

Studies conducted during outbreaks cannot be planned in advance, and the sample size is not predetermined, often resulting in a low statistical power for detecting significant associations between exposure and disease. In our study, the sample size (n = 83) reached a statistical power equal to 31% to detect as significant a PPR ≥ 2 (a total sample size of 275 would be needed to reach a statistical power equal to 80%).

Asking case-patients to identify the persons from whom they had probably acquired the disease could have introduced a bias because it implied a preconceived idea about how the infection was transmitted. However, most of the case-patients indicated that sick relatives whom they had cared for were their index patients; moreover, all of these index patients who were tested had positive results for Ebola antigens or antibodies. Both of these facts suggest that this bias did not significantly affect the results. Moreover, the retrospective design of the study, conducted in an emergency situation and in part based on a surveillance system that was created only in the third week of October, may have made it easier to identify sick contacts, as opposed to healthy contacts, because of the effect of recall bias. For this reason, we decided not to calculate the secondary attack rate or the reproductive rate. Furthermore, the group of uninfected contacts may have been inadvertently selected in a biased manner. Finally, the fact that the information was in most cases obtained from proxies may have also affected the results regarding risk factors. However, these results contribute to the knowledge on the patterns of transmission and risk factors for EHF, which is fundamental for better controlling new outbreaks.

The results of this study stress the importance of early detection and isolation of EHF patients in hospitals and the use of strict barrier nursing precautions in successfully controlling EHF outbreaks. Our findings on risk factors can also contribute to the efforts for educating communities and preventing the spread of the disease.

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Hantavirus Pulmonary Syndrome, Southern Chile

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We analyzed data from 25 consecutive patients with hantavirus pulmonary syndrome (HPS) admitted to the Puerto Montt and Osorno Regional Hospitals, southern Chile, from 1997 to 2001, emphasizing epidemiologic, clinical, radiographic, treatment, and laboratory aspects. Hemorrhage was frequent (64%), and 48% of patients showed alterations in renal function. Ten patients died (40%). We identified three groups of patients, which included the following: 1) those with the least severe form who had prodromic symptoms without pulmonary involvement; 2) those with moderate illness who had interstitial pulmonary infiltrates, usually needed supplemental nasal oxygen, were hemodynamically stable, and had an APACHE II <12 (none of whom died); and 3) those with the severe form who required mechanical ventilation, frequently had hemodynamic instability (93%), experienced a high mortality rate (77%), and had an APACHE II >12. Mild forms of HPS also exist, which are poorly known; the symptoms could be confounded with those of other viral diseases, leading to underdiagnosis.

Human infection and disease caused by *Hantavirus* spp. were unknown in the Americas until May 1993 (1). Since then, hantavirus infection has been reported in the United States, Brazil, Paraguay, Bolivia, and Chile (2–5). A new species called Andes virus was isolated during an outbreak in Argentina, with a rodent reservoir of *Oligoryzomys longicaudatus* in rural Argentina and Chile (6).

The most well-known clinical presentation of hantavirus pulmonary syndrome (HPS) starts with an influenzalike stage, with high fever, myalgia, asthenia, and after a prodromal period of 2 to 7 days, dyspnea, respiratory failure, and hemodynamic instability. Chest radiography shows rapidly progressing bilateral interstitial infiltrates, with an elevated hematocrit and thrombocytopenia. Other signs and symptoms have been reported, and in some case-patients, the disease may not progress beyond the prodromal stage or clinical symptoms may be completely absent (7).

Asian and European forms of hantavirus disease consist of a group of febrile nephropathies known as hemorrhagic fever with renal syndrome (8), clinical features of which differ from the forms described in the Americas. This study describes some aspects of this emergent disease in a group of 25 patients with confirmed hantavirus infection, emphasizing clinical, radiographic, and laboratory aspects.

Materials and Methods

Patient Population

Clinical chart information was recorded from of all patients with hantavirus pulmonary syndrome (HPS) admitted to the Osorno (n = 7) and Puerto Montt (n = 18) Hospitals from 1997 to 2001. All cases were confirmed by serologic tests performed at the virology laboratories of the Public Health Institute (Santiago) or Universidad Austral (Valdivia), with enzyme-linked immunoassay (ELISA) for immunoglobulin (Ig) M and IgG antibodies using Sin Nombre virus antigens provided by the Centers for Disease Control and Prevention, Atlanta, Georgia, USA. The Student t test was used to compare parametric variables, and chi square and Fisher exact test were used to compare discrete variables when necessary. A p value <0.05 was considered statistically significant.

Data Collection

The following data were recorded: age, sex, work type, residence, probable mechanism of infection, incubation period (only for those case-patients for whom precise information on the time of rodent exposure and onset of symptoms was available), medical history, and differential diagnosis. On admission, dyspnea, fever, anorexia, asthenia, headache, myalgia, chills, cough, abdominal pain, cyanosis, abnormal breathing sounds, hypotension (systolic blood pressure [SBP] <100), pulse rate, temperature, and respiratory frequency were recorded. A pseudoinfluenza course was understood to mean that the patients had a high

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fever, myalgia, headache, asthenia, and usually no rhinorrhea.

Clinical records noted any bleeding, alterations in renal or hepatic functions, alteration in consciousness, intensive care unit (ICU) admission, oxygen support, arterial oxygen tension (PaO_2)/inspiratory oxygen fraction (FiO_2) (PAFI), administration of antibiotic drugs or corticosteroids, and mechanical ventilation (duration and time of connection). We defined a renal function abnormality according to American Thoracic Society (ATS) criteria with plasma creatinine values at the onset or during the course of the disease >1.2 mg/dL or blood urea nitrogen [BUN] >20 mg/dL (9), which are considered specific risk factors for death or a complicated course of community-acquired pneumonia.

Chest x-rays were analyzed, and the results were classified as an alveolar, interstitial, or mixed pattern, and the distribution of radiographic infiltrates as unilobar or multilobar, and unilateral or bilateral. The presence of pleural effusion was noted. In addition, we looked for an increase in the size of the opacity by $\geq$50% within 48 hours of admission because a rapid spreading of infiltrates shown by radiograph (which usually cannot be determined at the time of admission) indicates severe community-acquired pneumonia (9).

Laboratory tests at admission and during the course of the illness were recorded.

Methylprednisolone was administered (to 10 patients) according to a published protocol (10): 1,000 mg IV per day for 3 days, followed by 16 mg orally per day for 3 days, 8 mg per day for 3 days, and finally, 4 mg per day for 3 days.

An APACHE II severity score was calculated for every patient (11). For those admitted to the ICU, the score was calculated by using the data recorded at that time. For those patients not admitted to ICU, we used the worst parameters during hospitalization to find the worst APACHE.

Shock was defined as having a systolic blood pressure of <90 (which was not changed by fluid administration or required the use of vasoactive drugs), or as abnormalities in tissue perfusion shown by state of consciousness, oliguria, lactate acidosis, or both (12). Refractory shock was defined as shock lasting >1 hour with no response to volume resuscitation or pharmacologic therapy (13). We considered hemodynamic instability to have occurred when hypotension (SBP <100), fitting or not fitting the shock criteria, took place at any time during the clinical course. The length of hospital stay and the mortality rate were also evaluated.

Results

The data of 25 patients with confirmed cases of HPS were analyzed (17 men, 8 women, mean age 33.4 years, range 15-63 years). The infection was most commonly acquired through farm or timber work (40%), and 76% of the patients were rural residents. The incubation period, estimated in 14 cases, was 9.8 $\pm$ 7.5 days (range 3-28 days). Symptom duration before admission was 5 $\pm$ 1.8 days (range 2-10 days); 12 (48%) of 25 patients had requested previous medical care on one to four occasions before admission; and in 20 (80%) of 25 case-patients, a diagnosis other than HPS was initially suggested.

The differential diagnoses before HPS was considered were community-acquired pneumonia or pneumonitis (including viral, interstitial, atypical, *P. carinii* pneumonia) (9 patients), sepsis (with/without shock or acute respiratory distress syndrome) (5 patients), fever syndrome (4 patients), influenza (3 patients), acute abdominal condition (1 patient), acute pyelonephritis (1 patient), acute tonsillitis (1 patient), bacterial meningitis (1 patient), typhoid fever (1 patient), acute diarrhea (1 patient), and myeloproliferative syndrome (1 patient). The main clinical signs and symptoms at admission were fever in 24 (96%) of 25 patients, myalgia in 24 (96%), asthenia in 19 (76%), headache in 15 (60%), abnormal breathing sounds on auscultation in 15 (60%), abdominal pain in 13 (56%), anorexia in 12 (48%), dry cough in 10 (40%), chills in 8 (32%), vomiting or nausea in 6 (24%), cyanosis in 5 (20%), diarrhea in 2 (8%), hemoptoic sputum in 2 (8%), and epistaxis, metrorrhagia, generalized maculopapular rash, consciousness alteration, lumbar pain, and odynophagia, each in 1 patient. Pseudoinfluenza syndrome was found in 17 (68%) patients. Bleeding was documented in 16 (64%) of 25 patients, and 3 (12%) patients required medical care for this reason. Tables 1 and 2 show the type and severity of bleeding manifestations and their relationship to platelet count.

The results of laboratory tests at admission can be seen in Table 3; thrombocytopenia was indicated by a platelet count of <100,000 in 23 (92%) case-patients. The mean hematocrit values were 49.8 $\pm$ 6.4, and three patients showed a value <45%. Twelve (48%) patients showed alterations in renal function, six (24%) exhibited a creatinine level >2.0, and one patient required hemodialysis. In 18 (72%) patients, liver function tests showed alterations, but only one patient had liver failure.

Hypotension at admission was observed in 12 (48%) of 25 patients, and 16 (60%) had hemodynamic instability (reaching 93% in the most severe group). Eight patients experienced refractory shock. In one patient, a Swan-Ganz catheter was used in a late stage of the infection, and the hemodynamic profile was consistent with septic shock, with low systemic venous resistence and high cardiac output. These conditions were also consistent with a case of nosocomial gram-negative sepsis; this was later demonstrated by blood culture. This case was the only one in

Table 1. Bleeding in patients with hantavirus pulmonary syndrome

Area of bleeding	n = 16 (%)
Pulmonary	8 (32)
Hematuria	6 (24)
Puncture sites	5 (20)
Skin (petechiae)	3 (12)
Hematemesis	2 (8)
Gingivorrhagia	2 (8)
Metrorrhagia	2 (8)
Subarachnoid hemorrhage	1 (4)
Peridural (lumbar puncture)	1 (4)
Epistaxis	1 (4)
Subungual	1 (4)
Visceral (necropsy)	1 (4)

which a bacterial overinfection was suspected and confirmed.

Radiographic changes exhibited at admission consisted of bilateral interstitial infiltrates in 14 patients (56%), alveolar infiltrates in 4 (16%), and mixed infiltrates in 5 (20%) patients. In two (8%) patients, the disease progressed without pulmonary infiltrates. The pulmonary infiltrates, when present, were bilateral and extended, involving 4 or 5 lobules in all cases. In seven (28%) patients, radiographic infiltrates were found to progress after 48 hours. Two patients showed pleural effusion. Samples from two patients with no pulmonary involvement underwent serologic tests because the patients lived in a rural region where previous cases had been found and because they had a clinical history of fever, myalgia, and malaise. One patient had thrombocytopenia (platelet count 53,000), and both had raised hematocrit values (50% and 46 %).

The mean PAFI at admission was 160 $\pm$ 121 (range 40-508). Eight (32%) patients received oxygen either nasally or by mask, and in three (12%), supplemental oxygen was not required. Fifteen (60%) patients were admitted to the ICU, and 14 (56%) received mechanical ventilation early, 9 (64%) of 14 during the first 24 hours after admission, 4 before 48 hours, and 1 patient on day 3. The mean period of mechanical ventilation was 4 days (range 1-13 days). Ten (40%) patients died, seven before completing 1 day in the hospital, and one patient died later of septicemia caused by gram-negative bacteria. All patients who died showed diffuse and rapidly progressive interstitial pulmonary infiltrates, compatible with massive pulmonary edema, severe respiratory failure and refractory shock. Finally, electromechanical dissociation and asystolia were noted.

For the 15 patients admitted to the ICU, the mean APACHE II score was 18.3$\pm$8.7. No patient with APACHE II <12 died, and the mortality rate for the patients with APACHE II >12 was 77% (10/13) ($p = 0.000$). In the group of 10 case-patients who received methylprednisolone, 2 (20%) died, which contrasts with 8 (53%) deaths of 15 who did not receive this drug ($p = 0.21$).

We administered prednisolone to 10 patients; the decision to use it was made on admission for half of the case-patients and 2 to 4 days later for the remaining case-patients. One patient had hyperglycemia associated with the use of methylprednisolone. Nineteen (76 %) patients received antibiotic drugs, which were usually discontinued

Table 2. Hemorrhage in patients with hantavirus pulmonary syndrome

Case no.	Bleeding sites	Platelet count 10^3/UL	Bleeding severity[a]	Prothrombin (%)	Outcome
1	Hemoptysis, hematemesis, hematuria	15.6	Moderate	NA[b]	Survived
3	Hematuria, gingivorrhagia	74.0	Mild	100	Survived
4	Hematuria, subungueal, hemoptysis	33.0	Moderate	NA	Died
5	Puncture sites, skin, hemoptysis	19.0	Mild	74	Survived
6	Hematuria	7.1	Moderate	NA	Survived
7	Skin, hematuria, hemoptysis, puncture sites, gingivorrhagia	28.0	Severe	100	Died
9	Skin, hematemesis, hemoptysis, hematuria, subarachnoid	25.0	Severe	13	Died
10	Metrorrhagia	73.0	Severe	NA	Died
11	Hemoptysis	23.0	Moderate	NA	Died
12	Hemoptysis	46.0	Mild	100	Survived
13	Puncture sites	45.0	Mild	100	Survived
14	Puncture sites	11.2	Mild	100	Survived
15	Hemoptysis, epistaxis	20.0	Mild	100	Survived
16	Puncture sites, dura mater	52.0	Severe	100	Survived
20	Metrorrhagia	31.0	Severe	57	Died
21	Viscera	154.0	Mild	NA	Died

[a]Mild, <200 mL (self limited); moderate, 200–500 mL (no transfusion needed); severe $\geq$500 mL (transfusion necessary).
[b]NA, not available.

Table 3. Laboratory results at admission of 25 patients with hantavirus pulmonary syndrome

	N	Media ± SD	Minimum	Maximum
Blood pH	24	7.39 ± 0.09	7.09	7.51
PaO_2 (mm Hg)	24	65.04 ± 24.7	32	131
$PaCO_2$ (mm Hg)	24	27.12 ± 12.1	14	74
Hematocrit (%)	25	49.84 ± 6.4	39	69
Platelets (1,000//µL	24	53,850 ± 40,996	9,900	174,000
Leukocytes (1,000//µL)	25	16,612 ± 14,781	2,800	59,400
Band forms (%)	14	10.8 ± 7.0	2	23
Lymphoblasts (%)	11	22.3 ± 13.9	6	48
HSR (mm/h)	5	5.8 ± 6.9	0	16
Sodium (mEq/L)	23	130.8 ± 5.6	120	143
Potassium (mEq/L)	22	4.0 ± 0.7	3	5.6
Creatinine (mg/dL)	22	1.38 ± 0.5	0.7	3.1
Uremia (mg/dL)	6	76.7 ± 54.2	16	140
Prothrombin (%)	13	80.2 ± 26.9	13	100
Bilirubin (mg/dL)	13	0.69 ± 0.35	0.31	1.44
Alkaline pH (U/L)	9	141.6 ± 56.2	78	233
GOT (U/L)	12	211.8 ± 162.2	24	478
GPT (U/L)	3	43.6 ± 21.8	20	63

[a]SD, standard deviation; HSR, human sedimentation rate; GOT, glutamic-oxaloacetic transminase; GPT, glutamic-pyruvic transminase.

after confirmation of the viral cause. The variables with statistically significant differences between survivors versus nonsurvivors are shown in Table 4. Other variables associated with death were cyanosis (p = 0.005), renal function alteration (p = 0.001), shock (p = 0.000), ICU admission (p = 0.001), and mechanical ventilation (p = 0.001).

Statistically significant differences were not observed for the following variables: hematocrit, platelet count, number of symptomatic days before admission, PaO_2, leukocyte count, plasma sodium and potassium, bilirubin, glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT), type and number of bleeding episodes, alteration in consciousness, growth of 50% in radiographic images during the first 48 hours, and the use of methylprednisolone.

We identified three groups of case-patients. The first included two case-patients with only prodromic symptoms without pulmonary involvement. The second group (n = 9) consisted of patients who had a self-limited form of the disease, usually with different degrees of interstitial pulmonary infiltrates, who did not require intubation and received oxygen through noninvasive methods (including one case-patient who needed no oxygen), were hemodynamically stable, and had a low APACHE II (<12). The third group (n = 14) had severe forms of the disease with a high mortality rate (APACHE II >12), required intubation and mechanical ventilation, with hemodynamic instability in 13 (93%) and refractory shock in 8 (57%). Pulmonary infiltrates were usually alveolar. The mean hospital stay was 13.6 days ± 24.7, ranging from hours for those patients who died quickly to 120 days in one patient with a peridural hematoma and severe secondary paraparesia as a consequence of a lumbar puncture.

Discussion

Our study confirms the existence of different clinical forms of hantavirus disease (7), including some without pulmonary involvement. A higher mortality rate was clearly associated with the most severe form (APACHE >12) of the disease, and death frequently occurred within the first

Table 4. Variables with statically significant differences in survivors versus no survivors with hantavirus pulmonary syndrome[a]

	Survivors		No survivors		
	Mean ± SD	CI 95%	Mean ± SD	CI 95%	p
APACHE	7.1 ± 1.2	4.4 to 9.8	21 ± 8.3	8.3 to 15.0	0.0000
PAFI	211 ± 127	134 to 288	95 ± 78	39.5 to 151.3	0.0097
Blood pH	7.43 ± 0.04	7.40 to 7.45	7.32 ± 0.12	7.23 to 7.41	0.0043
Creatinine (mg/dL)	1.08 ± 0.23	0.94 to 1.23	1.8 ± 0.59	1.35 to 2.27	0.0003
Prothrombin (%)	90.9 ± 17.8	75.9 to 105.7	63.2 ± 32.0	23.4 to 102.9	0.0340
SBP (mm Hg)	103.7 ± 20.0	92.6 to 114.8	76.6 ± 47.3	42.7 to 110.4	0.0296
Respiratory rate	25.2 ± 10.2	19.5 to 30.8	38.4 ± 7.8	32.4 to 44.4	0.0015
Pulse	104.6 ± 16.5	95.4 to 113.8	117.7 ± 21.9	102 to 133.4	0.0512

[a]SD, standard deviation; CI, confidence interval; PAFI, arterial oxygen tension/inspiratory oxygen fraction; SBP, standard blood pressure.

24 hours of hospital admission (70%). The main variables associated with death were APACHE II score, low PAFI, cyanosis, polypnea and tachycardia at admission, alterations in renal function, shock, and mechanical ventilation.

The incubation period was estimated for 14 cases and ranged from 3 to 28 days ($x = 9.8 \pm 7.5$), which is consistent with other reports (7,14,15). The symptoms at admission were similar to those described in case-patients in the United States (16,17) and Chile (10,14). Fundamentally, the initial clinical presentation is similar to influenza without rhinorrhea, high fever, myalgia, headache, or asthenia, and often accompanied by abdominal symptoms such as pain, nausea, vomiting, and diarrhea. After a prodromal period of 2 to 7 days, respiratory symptoms appear with dyspnea, respiratory failure, and hemodynamic instability. Chest x-rays show bilateral interstitial infiltrates of different degrees of rapid progression.

Nevertheless, the infection can be manifested in other ways, and many cases do not go beyond the prodromal stage or elicit any symptoms. In January 1997, the first hantavirus serologic study was carried out in Chile; of 64 serum samples collected in a rural location, 7 were positive for specific Andes virus nucleoprotein IgG. These 64 samples represented almost the whole population living in the area of the first Chilean case-patient's residence. Thus nearly 10% of this rural population had had contact with the virus, although none of the persons who provided a sample remembered a severe illness (7).

From 1995 to 2000, a total of 10 case-patients with mild forms of hantavirus disease were detected in Chile. The disease progressed in these case-patients without major pulmonary involvement, but the available information does not allow a close analysis of the clinical features of these cases, since most findings were from epidemiologic and serologic investigation (18). We found two case-patients who were admitted to the hospital because of a clinical course of fever, myalgia, and malaise, whose disease progressed with no pulmonary involvement as indicated by serial x-rays during their hospital stay. The diagnosis was suggested after considering the epidemiologic data (they lived in a rural area from which other cases had come) and suggestive laboratory test results: thrombocytopenia indicated by a platelet count of 53,000 in one and raised hematocrit value in both (50% and 46%). In these patients, the symptoms could have been confounded with a viral disease such as influenza and thereby been underdiagnosed because of the nonspecific character of the symptoms. In 80% of our case-patients, the initial diagnosis was incorrect, and 48% had received medical care on one to four occasions before hospital admission. By asking epidemiologically oriented questions in these endemic countries and regions, physicians may be able to make an earlier diagnosis of HPS.

In the series of patients described by Duchin et al. (16) in the United States, the early symptoms were similar to those described in the Asian and European forms of hantavirus disease known as hemorrhagic fever with renal syndrome. Nevertheless, no patient showed hemorrhages, renal involvement was minimal, and neither oliguria nor renal failure was observed in any case. The 1995 outbreak in Argentina was described as similar but details were not given (19). By contrast, the cases described in Chile are different, given the hemorrhagic manifestations observed. In Coyhaique (Chile), Tapia (10) saw petechiae in 38% (9/24), epistaxis in three pediatric patients and microhematuria in 16 patients (66.6%). In Region IX of Chile, Castillo (14,15) described a higher frequency of bleeding events (71% and 81%), including hematemesis, hemoptisis, epistaxis, and puncture site bleeding, similar to what we observed in 64% of the patients (Tables 1 and 2). In three patients of our series, a bleeding event was the reason for seeking healthcare. Thus, hemorrhagic manifestations appear to occur frequently in cases of hantavirus in Chile.

Concerning renal function, Tapia (10) observed raised creatinine of $\geq$1.5 in 7 of 24 cases, which did not correlate with the severity of clinical course. In this study, acute renal failure was considered a terminal phenomenon. Castillo reported two case-patients with acute renal failure; one required hemodialysis, and creatinine values were elevated (1.2-3.3 mg/dL) in 54% of the patients (14,15). In our series, 48% of the patients fulfilled the American Thoracic Society criteria for altered renal function (9), and the difference between the creatinine values of nonsurvivors and survivors measured at admission ($p = 0.0003$) was statistically significant. In 24%, creatinine values were >2.0, and one patient required hemodialysis. We believe that the use of common criteria to define renal failure in larger series can help estimate the changes in renal function, which seem to be more important than previously described and have a prognostic value. With regard to laboratory tests, raised hematocrit values and low platelet counts are exhibited by almost all patients at admission, and thus are important diagnostic elements, although they have no prognostic value.

Corticosteroids have the potential of modulating intrapulmonary inflammatory response by modifying the proinflammatory cytokine levels such as interleukin-1-β and tumor necrosis factor-∝ (TNF-∝), and have been used in the management of severe pulmonary disorders, with both an infective as well as a noninfective etiology such as miliary tuberculosis, *Pneumocystis carinii* pneumonia, vasculitides, and gastric acid aspiration (20).

In our study, 2 of 10 patients who received methylprednisolone (20%) died versus. 8 of 15 (53%) in the group who did not receive this drug, although this difference was not statistically significant (p = 0.21). In larger series, a significant effect could perhaps be demonstrated. When the first cases were being diagnosed, however, we did not consider the use of corticosteroids; no protocol on how to use them existed, and most severely ill patients died very quickly and thus did not receive corticosteroids; their usage probably would not have changed the fatal course of the disease in this group, however.

The mortality rate in our series was directly related to the type of clinical presentation. In the severe form, with APACHE II >12, the mortality rate reached 77%, in contrast to those with a score <12, among whom no deaths were observed. From 1993 to July 2000, a total of 123 confirmed HPS cases were reported in Chile, 61 (49.6%) of which were fatal. This rate has decreased during the last 2 years, partly because of the improvement in diagnostic capacities and greater knowledge of the disease, which allows quicker identification and more effective treatment (18), but we think that this reduction also may be due to a dilution effect because more mild cases are found.

In our series, we distinguish three groups of patients. The first (n = 2), probably the least known, corresponds to patients with only prodromal symptoms, especially a pseudoinfluenza course with no pulmonary involvement, who are often not admitted to a hospital and are not recognized to have HPS. Correct diagnosis of these cases improves if one makes a good epidemiologic questionnaire. Groups 2 and 3 allow the patients to be classified according to clinical and radiologic criteria, severity, treatment, and prognosis. Use of uniform criteria is required to classify the patients infected by hantavirus with different clinical presentations, to thereby comparatively evaluate the mortality rate and the effectiveness of therapeutic measures.

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Triosephosphate Isomerase Gene Characterization and Potential Zoonotic Transmission of *Giardia duodenalis*

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To address the source of infection in humans and public health importance of *Giardia duodenalis* parasites from animals, nucleotide sequences of the triosephosphate isomerase (TPI) gene were generated for 37 human isolates, 15 dog isolates, 8 muskrat isolates, 7 isolates each from cattle and beavers, and 1 isolate each from a rat and a rabbit. Distinct genotypes were found in humans, cattle, beavers, dogs, muskrats, and rats. TPI and small subunit ribosomal RNA (SSU rRNA) gene sequences of *G. microti* from muskrats were also generated and analyzed. Phylogenetic analysis on the TPI sequences confirmed the formation of distinct groups. Nevertheless, a major group (assemblage B) contained most of the human and muskrat isolates, all beaver isolates, and the rabbit isolate. These data confirm that *G. duodenalis* from certain animals can potentially infect humans and should be useful in the detection, differentiation, and taxonomy of *Giardia* spp.

Giardiasis is a common cause of diarrheal disease in almost all vertebrates, including humans. In industrialized countries, it is referred to as a reemerging infectious disease because of its increasingly recognized role in outbreaks of diarrheal disease in daycare centers and in water- and foodborne outbreaks. *Giardia* is also one of the most frequently observed parasites infecting dairy cattle and domestic dogs. In developing countries in Asia, Africa, and Latin America, approximately 200 million people have symptomatic giardiasis (1).

The taxonomy of *Giardia* at the species level is complicated and unresolved because of limited morphologic differences. Based on morphology, six species of this genus are considered valid: *Giardia duodenalis* (syn. *G. lamblia* or *G. intestinalis*) in a wide range of mammals, including humans, livestock, and companion animals; *G. agilis* in amphibians; *G. muris* in rodents; *G. ardeae* and *G. psittaci* in birds; and *G. microti* in muskrats and voles (2–6). However, on the basis of host origins, 41 *Giardia* species have been named (7,8).

Molecular tools have been used recently to characterize the epidemiology of human giardiasis. Although isolates of *G. duodenalis* from humans and various animals are morphologically similar, distinct host-adapted genotypes have been demonstrated within *G. duodenalis* (1,9–12). Two major groups of *G. duodenalis* have been recognized as infecting humans worldwide, but there are some differences in naming of these groups, as evidenced by the following categorizations: Polish and Belgian genotypes (9); groups 1, 2, and 3 (10,13); and assemblages A and B (11). So far, no general consensus has been reached concerning the nomenclature of these genotypes, but the term assemblages has been more widely used. The finding of host-adapted *Giardia* genotypes is of public health importance, considering the controversy regarding the zoonotic potential of *Giardia* (1,14).

We describe the development of a two-step nested polymerase chain reaction (PCR) protocol to amplify the triosephosphate isomerase (TPI) gene of *G. duodenalis* and *G. microti* and nucleotide sequence characterization of amplified TPI fragment. The TPI gene was chosen because of the high genetic heterogeneity displayed by *Giardia* spp. at this locus (12,15). Results of the study have validated previous observations on the genetic diversity of *Giardia* parasites on the basis of characterization of the glutamate dehydrogenase (GDH), small subunit ribosomal RNA (SSU rRNA), and TPI genes (12,15–17). These data also suggest that some animal isolates of *G. duodenalis* are

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of zoonotic potential. These data should be useful in developing alternative molecular tools to differentiate *Giardia* parasites at species and genotype levels and in investigating giardiasis outbreaks or endemic diseases.

Materials and Methods

G. duodenalis Isolates and DNA Extraction

Fecal samples containing *G. duodenalis* cysts were obtained from infected humans, cattle, companion animals (dogs and a rabbit), aquatic wildlife (beavers and muskrats), and one rat. Human samples were mostly from sporadic cases, with the exception of two isolates (4599, 4600) from a foodborne outbreak. Fecal samples with *G. microti* were obtained from infected muskrats. *Giardia* infection was diagnosed by microscopy of wet mounts or immunofluorescence-stained materials. Samples were stored at 4°C in 2.5% (w/v) potassium dichromate solution or frozen at –20°C and used in DNA extraction without cyst isolation (Table 1). For DNA extraction, 200 µL of the fecal suspension from each sample was aliquoted and washed three times with distilled water. The material was treated initially with 66.7 µL of 1 M KOH and 18.6 µL of 1 M dithiothreitol (DTT) followed by neutralization with 8.6 µL of 25% (v/v) hydrochloric acid. The DNA lysate was then extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) solution, and purified by using the QIAamp DNA Stool Kit (QIAGEN Inc, Valencia, CA). Because the extracted DNA contained nucleic acids from both *Giardia* cysts and fecal materials, DNA concentration was not determined for all samples.

PCR Amplification of the TPI Gene

To amplify the TPI fragment from various *Giardia* isolates, a nested PCR protocol was developed that used primers complementary to the conserved published TPI nucleotide sequences of various *Giardia* parasites downloaded from GenBank: *G. duodenalis* (U57897, AF06957 to AF069563, L02116, L02120), *G. muris* (AF069565), and *G. ardeae* (AF069564). For the primary PCR, a PCR product of 605 bp was amplified by using primers AL3543 [5′-AAATIATGCCTGCTCGTCG-3′] and AL3546 [5′-CAAACCTTITCCGCAAACC-3′]. The PCR reaction comprised 0.25–2.0 µL of DNA, 200 µM each of deoxynucleoside triphosphate (dNTP), 1X PCR buffer (Perkin Elmer, Wellesley, MA), 3.0 mM $MgCl_2$, 5.0 U of *Taq* polymerase (GIBCO BRL, Frederick, MD), and 200 nM of each primer in a total of 100-µL reaction. The reactions were performed for 35 cycles (94°C for 45 s, 50°C for 45 s, and 72°C for 60 s) in a Perkin-Elmer GeneAmp PCR 9700 thermocycler, with an initial hot start (94°C for 5 min) and a final extension (72°C for 10 min). For the secondary PCR, a fragment of 530 bp was amplified by using 2.5 µL of primary PCR reaction and primers AL3544 [5′-CCCTTCATCGGIGGTAACTT-3′] and AL3545 [5′-GTGGCCACCACICCCGTGCC-3′]. The conditions for the secondary PCR were identical to the primary PCR. The PCR products were analyzed by agarose gel electrophoresis and visualized after ethidium bromide staining.

PCR Amplification of the SSU rRNA Gene

A nested PCR protocol was also developed to amplify the SSU rRNA fragment from *Giardia* isolates, using primers complementary to the conserved published SSU rRNA nucleotide sequences from various *Giardia* parasites downloaded from GenBank: *G. duodenalis* (AJ278959, AJ293295 to AJ293299, AJ293300, AJ293301, L29129, M54878, U09491, U09492, X52949), *G. microti* (AF006676, AF006677), *G. muris* (X65063), and *G. ardeae* (Z17210). For the primary PCR, a PCR product of 300 bp was amplified by using primers AL4303 [5′-ATCCGGTCGATCCTGCCG-3′] and reverse AL4305 [5′-AGGATCAGGGTTCGACT-3′]. The PCR reaction was performed by using the GC-RICH PCR System kit, which consisted of GC-RICH Enzyme mix (Taq polymerase in combination with a proofreading polymerase), GC-RICH-PCR reaction buffer (includes a final 1.5 mM $MgCl_2$ and dimethyl sulfoxide [DMSO]), and GC-RICH resolution solution (Roche Diagnostics, Indianapolis, IN) with 0.25–2.0 µL of DNA, 200 µM each of dNTP, and 200 nM of each primer in a total of 50-µL reaction. For the secondary PCR, a fragment of 255 bp was amplified with the GC-RICH PCR System kit (Roche) with 2.5 µL of primary PCR reaction, and 200 nM of primers AL4304 [5′-CGGTCGATCCTGCCGGA-3′] and AL4306 [5′-GGCGGAGGATCAGGGT-3′]. The cycling conditions for both SSU RNA primary and secondary PCR were identical to those used to amplify the TPI gene.

DNA Sequencing and Phylogenetic Analysis

The secondary PCR products were purified by using Microcon PCR Centrifugal Filter Devices (Millipore Corp., Bedford, MA) and sequenced on an ABI 3100 automated sequencer by using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequence accuracy was confirmed by two-directional sequencing of two separate PCR products. Multiple alignment of the nucleotide sequences was performed by using Wisconsin Package Version 9.0 program (18). A phylogenetic analysis was performed on the aligned sequences to assess the extent of genetic diversity within *G. duodenalis* parasites as well as their evolutionary relationships with other *Giardia* species. In this analysis, published TPI nucleotide sequences representing *G. duodenalis* (from humans, cattle, cat, dog, muskrat, pig, and rat), *G. muris*, and *G. ardeae*

Table 1. *Giardia* isolates with genotype identity

Isolate	Location	Y	Host	Genotype/species	G+C content (%)
2875	Lima, Peru	2001	Human	Assemblage A	56.3
2891	Lima, Peru	2001	Human	Assemblage A	56.2
2893	Lima, Peru	2001	Human	Assemblage A	56.3
2905	Lima, Peru	2001	Human	Assemblage A	56.1
2907	Lima, Peru	2001	Human	Assemblage A	56.3
2922	Lima, Peru	2001	Human	Assemblage A	56.1
341	Hyderabad, India	1998	Human	Assemblage B	52.1
2578	Calcutta, India	2000	Human	Assemblage B	51.7
2579	Calcutta, India	2000	Human	Assemblage B	51.7
2580	Calcutta, India	2000	Human	Assemblage B	51.6
2582	Calcutta, India	2000	Human	Assemblage B	51.2
2583	Calcutta, India	2000	Human	Assemblage B	51.4
2586	Calcutta, India	2000	Human	Assemblage B	51.6
2587	Calcutta, India	2000	Human	Assemblage B	51.9
2589	Calcutta, India	2000	Human	Assemblage B	51.7
2590	Calcutta, India	2000	Human	Assemblage B	51.7
2506	Lima, Peru	2000	Human	Assemblage B	51.7
2536	Lima, Peru	2000	Human	Assemblage B	51.7
2877	Lima, Peru	2001	Human	Assemblage B	51.3
2879	Lima, Peru	2001	Human	Assemblage B	51.9
2887	Lima, Peru	2001	Human	Assemblage B	51.5
2890	Lima, Peru	2001	Human	Assemblage B	51.9
2895	Lima, Peru	2001	Human	Assemblage B	51.9
2900	Lima, Peru	2001	Human	Assemblage B	51.4
2901	Lima, Peru	2001	Human	Assemblage B	51.1
2902	Lima, Peru	2001	Human	Assemblage B	52.1
2913	Lima, Peru	2001	Human	Assemblage B	51.5
2915	Lima, Peru	2001	Human	Assemblage B	51.5
2917	Lima, Peru	2001	Human	Assemblage B	51.5
2920	Lima, Peru	2001	Human	Assemblage B	51.6
2924	Lima, Peru	2001	Human	Assemblage B	51.9
2926	Lima, Peru	2001	Human	Assemblage B	51.9
2930	Lima, Peru	2001	Human	Assemblage B	51.1
2932	Lima, Peru	2001	Human	Assemblage B	51.5
2935	Lima, Peru	2001	Human	Assemblage B	51.6
4599	San Francisco, CA	2001	Human	Assemblage B	51.9
4600	San Francisco, CA	2001	Human	Assemblage B	51.8
1758	Changchun, China	2000	Rabbit	Assemblage B	50.7
1653	Preston, MD	2000	Beaver	Assemblage B	50.7
1654	Preston, MD	2000	Beaver	Assemblage B	50.7
1655	Preston, MD	2000	Beaver	Assemblage B	50.9
3495	Preston, MD	2001	Beaver	Assemblage B	50.5
3500	Preston, MD	2001	Beaver	Assemblage B	51.1
3518	Preston, MD	2001	Beaver	Assemblage B	51.1
3599	Preston, MD	2001	Beaver	Assemblage B	50.7
3469	Preston, MD	2001	Muskrat	Assemblage B	50.9
3470	Preston, MD	2001	Muskrat	Assemblage B	50.9
3565	Preston, MD	2001	Muskrat	Assemblage B	50.9
3569	Preston, MD	2001	Muskrat	Assemblage B	50.7
3577	Preston, MD	2001	Muskrat	Assemblage B	50.1
867	Atlanta, GA	1999	Dog	Assemblage C	56.1
868	Atlanta, GA	1999	Dog	Assemblage C	56.4
894	Atlanta, GA	1999	Dog	Assemblage C	56.4
895	Atlanta, GA	1999	Dog	Assemblage C	55.5
898	Atlanta, GA	1999	Dog	Assemblage C	56.4
2643	Atlanta, GA	1999	Dog	Assemblage C	56.2

Table 1 continued. *Giardia* isolates with genotype identity

Isolate	Location	Y	Host	Genotype/species	G+C content (%)
2645	Atlanta, GA	1999	Dog	Assemblage C	55.8
2661	Atlanta, GA	1999	Dog	Assemblage C	55.9
2664	Atlanta, GA	1999	Dog	Assemblage C	56.4
2665	Atlanta, GA	1999	Dog	Assemblage C	55.8
2668	Atlanta, GA	1999	Dog	Assemblage C	56.2
2669	Atlanta, GA	1999	Dog	Assemblage C	56.2
2670	Atlanta, GA	1999	Dog	Assemblage C	56.1
2674	Atlanta, GA	1999	Dog	Assemblage C	55.8
2679	Atlanta, GA	1999	Dog	Assemblage C	56.6
15	Columbus, OH	1997	Cattle	Assemblage E	50.4
109	Columbus, OH	1997	Cattle	Assemblage E	50.6
110	Columbus, OH	1997	Cattle	Assemblage E	50.5
111	Columbus, OH	1997	Cattle	Assemblage E	50.6
112	Columbus, OH	1997	Cattle	Assemblage E	50.9
138	Columbus, OH	1997	Cattle	Assemblage E	50.8
5009	Beltsville, MD	2001	Cattle	Assemblage E	50.5
2135	St Louis, MO	2000	Rat	Assemblage undefined	58.6
3460	Preston, MD	2001	Muskrat	*G. microti*	57.9
3463	Preston, MD	2001	Muskrat	*G. microti*	58.2
3464	Preston, MD	2001	Muskrat	*G. microti*	50.9

were aligned with TPI sequences of *Giardia* parasites obtained in this study.

A neighbor-joining tree (19) was constructed on the basis of the evolutionary distances calculated by the Kimura-2-parameter model using the TreeconW program (20). A sequence of *G. ardeae* (GenBank accession no. AF069564) was used as the outgroup since the construction of an unrooted tree showed it to be the most divergent member under analysis. The reliability of these trees was assessed by using the bootstrap method (21) with 1,000 pseudoreplicates; values >70% were reported (22). Nucleotide sequences of the TPI gene of *G. duodenalis* from humans, cattle, dogs, muskrat, rat, and rabbit, representing different genotypes, were deposited in GenBank under accession numbers AY228628 to AY228649.

A similar phylogenetic analysis was carried out on the nucleotide sequences of the SSU rRNA gene from *G. microti* in muskrats. SSU rRNA nucleotide sequences were deposited in GenBank under accession numbers AY228332 and AY228333.

Results

PCR products of the expected size (approximately 500 bp) were generated from all 76 isolates. All were sequenced, and all of the nucleotide sequences obtained belonged to the TPI sequences of *Giardia* based on BLAST search of the GenBank database. The sources of these isolates were humans (37 isolates), dogs (15 isolates), muskrats (8 isolates), cattle (7 isolates), beavers (7 isolates), rabbit (1 isolate) and rat (1 isolate). The TPI gene of *Giardia* parasites was rich in GC content, ranging from 50.1% to 58.2% (Table 1). Isolates within each genotype, however, had very similar GC contents in the TPI gene.

The extent of genetic diversity in the genus *Giardia* was assessed by multiple alignments of the TPI nucleotide sequences followed by estimates of genetic distances (Table 2). The analysis showed distinct sequences for the human, cattle, beaver, dog, muskrat, and rat isolates; most animals had one genotype, and humans and muskrats had two genotypes. The genetic polymorphism in *Giardia* parasites was evident along the entire TPI gene both at the interspecies (*Giardia* spp.) and intraspecies (*G. duodenalis*) levels.

To understand the genetic structure of *Giardia* parasites, a neighbor-joining tree was constructed in a phylogenetic analysis of aligned TPI gene sequences of various *Giardia* species and *G. duodenalis* genotypes; we used the nucleotide sequence of *G. ardeae* (AF069564) as an outgroup to root the tree (Figure 1). The phylogenetic analysis showed four distinct clusters for the genus *Giardia*. The first cluster consisted of all isolates of *G. duodenalis* from various sources (humans, cattle, cats, dogs, beavers, muskrats, pigs, and rats). The second cluster consisted of some of the isolates from muskrats. The third and fourth cluster was each represented by a single published sequence of *G. muris* (AF069565) and *G. ardeae* (AF069564).

Several large groups were in the *G. duodenalis* cluster. A major group (assemblage B) was formed with most of the human and muskrat isolates, all the isolates from beavers, and the rabbit isolate (Figure 1). The remaining human isolates aligned with other previously reported human TPI sequences and formed a distinct cluster (assemblage A). Distinct clusters were also evident for the

Table 2. Evolutionary genetic distances between different *Giardia* species and *Giardia duodenalis* assemblages

	G. ardeae	*G. muris*	Undefined cat	Assemblage A	Assemblage E	Undefined rat	Assemblage C	Assemblage B	*G. microti*
G. ardeae	0.00	19.25	43.08	45.31	52.46	46.85	46.49	50.41	32.28
G. muris		0.00	46.53	47.48	47.40	47.38	47.14	47.40	44.26
undefined cat			0.00	10.53	12.82	19.16	22.40	24.34	32.23
Assemblage A				0.00	12.85	17.31	19.14	22.31	30.73
Assemblage E					0.00	23.07	22.35	25.54	36.88
undefined Rat						0.00	16.57	20.49	32.03
Assemblage C							0.00	21.69	27.72
Assemblage B								0.00	34.77
G. microti									0.00

isolates from dogs (assemblage C) and rats (undefined). The cattle sequences, together with the published pig TPI sequence, also formed a distinct cluster (assemblage E or hoofed livestock genotype). Phylogenetic analysis indicated that assemblages B and C and the rat genotype were related to each other and that assemblages A and E and the cat genotype were related to each other. The formation of all major groups was supported by bootstrap analysis with full statistical reliability (Figure 1).

Intragenotypic variations were evident within assemblages A, B, C, and E (Table 3). A very high degree of polymorphism was noticed within the isolates from humans. The human isolates grouped in assemblage B had five SNPs (single nucleotide polymorphisms): A or G at position 39, C or T at position 91, G or A at position 162, C or T at position 165, and C or T at position 168 (position numbers according to the GenBank accession no. L02116). Within assemblage B, 12 subtypes of *G. duodenalis* were noticed; 11 of these had not been reported before (Figure 2). No genetic polymorphism was evident in the TPI sequences of the beaver isolates characterized so far, which were identical to those from most muskrats belonging to the major assemblage B group. However, two muskrat isolates (3565, 3569) in assemblage B had one SNP at position 216 (C to T). Six SNPs (A or G at position 51, T or C at position 77, T or G at position 150, C or T at position 330, T or C at position 383, and C or A at position 393) were evident within the dog isolates (assemblage C). Multiple alignments of sequences from hoofed livestock showed two distinct subtypes in cattle with four SNPs (T or C at position 72, G or T at position 78, T or C at position 93, and G or A at position 109). The sequence from the rat matched with the TPI sequence from another suckling mouse (GenBank accession no. AF069562) with one SNP (G to A) at position 54. No genetic variation was observed in the human TPI sequences of assemblage A generated in this study, even though three sequences from GenBank (AF069556, L02120, and U57897) had three SNPs.

Since TPI nucleotide sequences of three isolates from muskrats were very different from known *G. duodenalis* isolates or with *C. muris* or *C. ardeae* and since they formed a distinct cluster, these isolates were characterized at the SSU rRNA locus. The *Giardia* SSU rRNA sequences obtained were aligned with the published sequences. Analysis showed that these isolates were *G. microti*. Of the three muskrat SSU rRNA sequences from this study, two (isolates 3460 and 3464) were identical to a published SSU rRNA sequence (AF006676) from muskrats (6). The third sequence (isolate 3463) was unique and had three SNPs compared with the other two muskrat isolates and AF006676. Isolate 3463 was still considered to be a

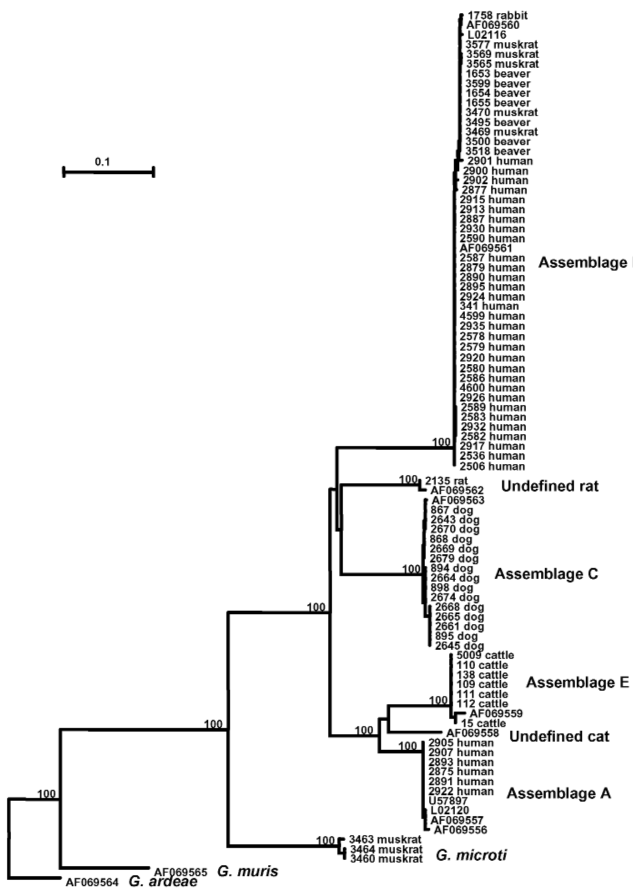

Figure 1. Phylogenetic relationships of *Giardia* parasites inferred by the neighbor-joining analysis of the triosephosphate isomerase (TPI) nucleotide sequences.

Table 3. Number of genotypes present in each assemblage

Assemblage	No. of isolates studied	No. of subtypes
A	6	1
B	44	12
C	15	4
E	7	2
Undefined rat	1	1
Giardia microti	3	2

sequence of *G. microti* because another published *G. microti* sequence (AF006677) was even more divergent (Figure 3a). A similar pattern of genetic polymorphism was evident in the TPI gene; the sequences from isolates 3460 and 3464 were identical to each other but had six SNPs compared with isolate 3463. This finding suggests that at least two distinct genotypes of *G. microti* were present in muskrats (Figure 3b).

Discussion

Understanding the taxonomic relationship of a particular group of protozoan parasites that truly reflects biological characteristics and evolutionary relationships is difficult. Most protozoan parasites lack fossil records, are microscopic, and have few informative morphologic and ultrastructural characters; some lack sexual reproduction (23,24). Although *Giardia* spp. populate the intestinal tracts of almost every group of vertebrates, *G. duodenalis* is the only species found in humans and many other mammals including cattle, cats, dogs, horses, sheep, and pigs (1,25,26). *Giardia* cysts have also been detected in various wild mammals (14,27–34). Although these wild mammals are generally assumed to be infected with *G. duodenalis*, molecular characterization to support this supposition is lacking.

Even though *Giardia* isolates from different mammalian hosts were similar in form, a marked biological diversity among these isolates was noticed in host infectivity (35), metabolism (36), and in vitro and in vivo growth requirements (37,38). Multilocus enzyme electrophoresis identified a number of distinct groups of *G. duodenalis* (39,40). The forgoing heterogeneity suggests that *G. duodenalis* is a species-complex (39,41,42). Phylogenetic characterization based on the nucleotide sequences of GDH, elongation factor 1α (EF1α), TPI, and SSU rRNA genes suggests the presence of five to seven lineages of *G. duodenalis* (12,17,43,44). Among the loci analyzed, TPI has the highest degree of polymorphism (12). However, only four isolates from humans, two isolates from mice,

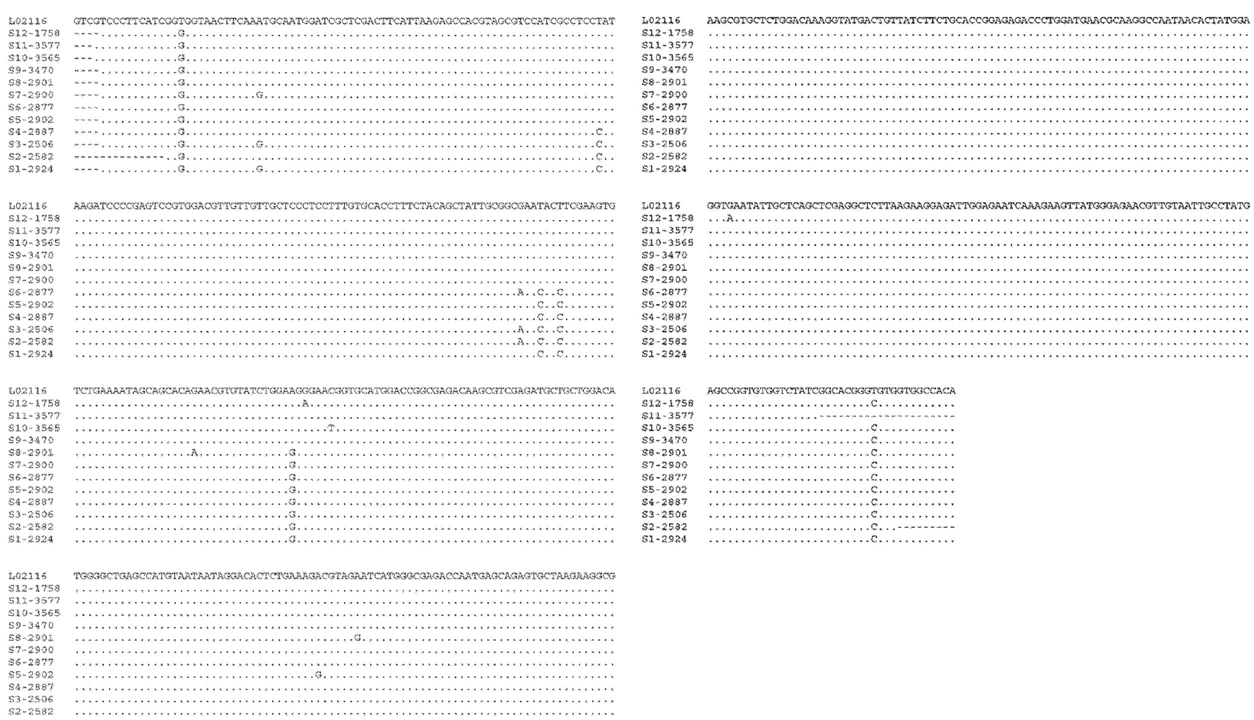

Figure 2. Variation in the triosephosphate isomerase (TPI) nucleotide sequences of *G. duodenalis* isolates belonging to the assemblage B. Twelve distinct subtypes of *G. duodenalis* based on the these sequences were evident within assemblage B. The isolates representing these subtypes (S1–S12) as follows: S1 (341, 2578, 2579, 2580, 2586, 2587, 2879, 2890, 2895, 2920, 2924, 2926, 2935, 4599, 4600); S2 (2582, 2583, 2589, 2932); S3 (2506, 2536, 2917); S4 (2590, 2887, 2913, 2915, 2930); S5 (2902); S6 (2877); S7 (2900); S8 (2901); S9 (1653, 1654, 1655, 3469, 3470, 3495, 3500, 3518, 3599); S10 (3565, 3569); S11 (3577); and S12 (1758). Dots denote sequence identity to GenBank accession no. L02116; dashes denote sequence information not obtained.

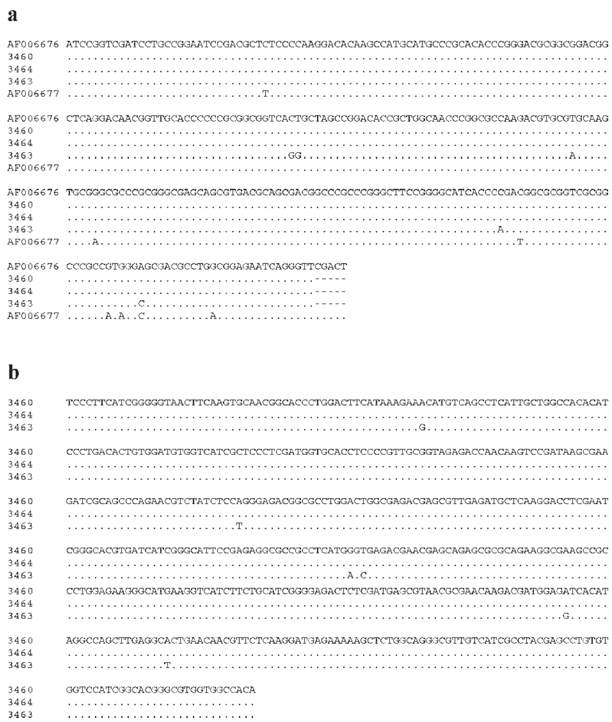

Figure 3. Genetic variation in the nucleotide sequences of *Giardia microti* parasites in the small subunit ribosomal RNA (SSU rRNA) (a) and triosephosphate isomerase (TPI) (b) genes.

and one isolate each from cat, dog, pig, rat (*G. muris),* and blue heron (*G. ardeae*) have been characterized at the TPI locus (12).

The genetic relationship among various *Giardia* parasites showed by phylogenetic analysis of the TPI gene in this study is largely in agreement with previous observations based on results from the SSU rRNA, TPI, GDH, and EF1α genes (12,17,43,44). Thus, on the basis of published and present TPI nucleotide sequences, the following groupings of *G. duodenalis* parasites are evident by all analyses with strong statistical reliability: 1) the formation of a group containing relatively few human isolates (assemblage A); 2) a major group containing most of the human and muskrats isolates, as well as isolates from beavers and a rabbit (assemblage B); 3) the formation of a group containing all isolates from cattle and pigs (assemblage E or the hoofed livestock genotype); 4) the formation of a group containing isolates from dogs (assemblage C); 5) an undefined cat genotype; and 6) an undefined genotype from rats. The assemblage D previously seen in a few dogs (42) was not found in this study.

In our study, a distinct and more distant cluster was formed by some isolates from muskrats. DNA sequence analysis of the SSU rRNA gene indicated that these isolates were *G. microti*. This organism was placed between the clades representing the *G. muris* and all the six assemblages of *G. duodenalis*. *Giardia microti* was established as a separate species because of sequence uniqueness of the SSU rRNA gene and minor morphologic differences from *G. duodenalis* (5,6). Our characterization of TPI nucleotide sequences from muskrats supports the validity of *G. microti*.

Results of phylogenetic analysis are useful in understanding the public health importance of some *G. duodenalis* parasites. Human *G. duodenalis* are placed in two distinct lineages (assemblages A and B), whereas the other four lineages contain only *G. duodenalis* from animals (assemblages C and E, and undefined cat and rat genotypes). One of the assemblages in humans, assemblage B, also contains all beaver isolates and some isolates from muskrats, rabbits, and mice, which strongly suggests that these animal isolates have the potential to infect humans. *Giardia* from beavers has been suggested as the source of infection for backpackers and some waterborne outbreaks of giardiasis (27,30). Results of our study provide genetic evidence to substantiate these claims.

The TPI-based genotyping tool is also useful in epidemiologic investigations of giardiasis in humans (15,45,46). A recent study in the United Kingdom of sporadic cases of human giardiasis used a TPI-based PCR–restriction fragment length polymorphism genotyping tool. Of the 33 TPI-PCR–positive infected patients, 21 (64%) were infected with assemblage B, 9 (27%) with assemblage A, and 3 (9%) samples were mixed infections of assemblages A and B (47). Similar results were obtained with samples from a nursery outbreak, in which 21 (88%) of 24 samples were shown to be *G. duodenlais* assemblage B parasites; the rest were assemblage A parasites (47). The intragenotypic variations of TPI in assemblage B identified in the present study should be useful in subtyping outbreak isolates. Because *Giardia* spp. have a clonal population structure (40), the use of a typing system based on sequence analysis of a single genetic locus with high sequence heterogeneity, such as TPI, can provide a resolution as high as multilocus sequence typing.

The results of our study suggest that the TPI gene is a good phylogenetic marker for analysis of the molecular evolutionary and taxonomic relationship of *G. duodenalis* parasites. The genetic relationship shown by phylogenetic analysis of the TPI gene is largely in agreement with that obtained at other genetic loci. Results of the molecular analyses support the conclusion that *G. duodenalis* is a species-complex, a finding that should be useful in the revision of *Giardia* taxonomy and standardization of *Giardia* nomenclatures. Results of this study also indicate that *Giardia* parasites from beavers, muskrats, mice, and rabbits represent a potential public health concern.

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Severe Acute Respiratory Syndrome–associated Coronavirus Infection

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Whether severe acute respiratory syndrome–associated coronavirus (SARS-CoV) infection can be asymptomatic is unclear. We examined the seroprevalence of SARS-CoV among 674 healthcare workers from a hospital in which a SARS outbreak had occurred. A total of 353 (52%) experienced mild self-limiting illnesses, and 321 (48%) were asymptomatic throughout the course of these observations. None of these healthcare workers had antibody to SARS CoV, indicating that subclinical or mild infection attributable to SARS-CoV in adults is rare.

The outbreak of severe acute respiratory syndrome (SARS) at the Prince of Wales Hospital, Hong Kong, began on March 10, 2003 (1,2). Within the next 10 weeks, the hospital admitted 331 patients with SARS; 160 (48.3%) were healthcare workers (HCWs). Prince of Wales is a 1,350-bed teaching hospital with 3,711 employees, of whom 12% are physicians, 36% nurses, 11% allied health workers, and the remainder, administrative and ancillary staff. During the outbreak, many HCWs had been exposed directly or indirectly to aerosols, body fluids, secretions, and excretions of SARS patients. The clinical manifestations of SARS are well documented (2–5). However, we do not yet know the spectrum of clinical disease or whether mild or asymptomatic infections attributable to the SARS-associated coronavirus (SARS-CoV) occur. Whether subclinical infections occur and whether one may seroconvert to the SARS-CoV with minimal or no symptoms are concerns for HCWs and others.

The Study

We performed a prospective study to determine whether asymptomatic or mild infection attributable to SARS-CoV was common in HCWs in this outbreak at Prince of Wales Hospital. When it had been established that an outbreak was occurring, a SARS screening clinic was instituted to care for hospital staff with symptoms suggestive of or suspected to be SARS. Asymptomatic staff or those without compatible symptoms were also invited to participate in this study. In late March and early April 2003, a blood sample was collected from each HCW who voluntarily participated and who wished to be tested for antibody to SARS-CoV; a second blood sample was collected 4–6 weeks later. Most of the second blood samples were collected in early May 2003, approximately 8 weeks from the first peak and 4 weeks from the second peak of admission of HCWs with SARS (Figure). Each HCW completed a questionnaire to document known direct contact with SARS patients, their body fluids, secretions, or excretions; places of duty within the hospital; and symptoms of any illness during the period between first and second blood sample collection. Additional information also included the department and the position of HCWs, so that the job nature could be delineated.

Immunoglobulin (Ig) G antibody to SARS-CoV was detected by an immunofluorescence assay on the basis of Vero cells infected with coronavirus isolated from a patient with SARS. We isolated this SARS-CoV and determined the complete genome sequence (GenBank accession no. AY278554). Serum samples were diluted 1:40 for antibody-screening assays. Each result was crosschecked by two experienced technicians. This immunofluorescence assay had been successfully used for serodiagnosis of SARS in patients in our hospital; titers of >320 developed in acutely ill SARS patients 4 weeks after onset of illness.

Conclusions

Six hundred and seventy-four HCWs completed the questionnaire and had a second serum sample obtained. The mean age of these HCWs was 40 years (range 20–60), and 75% were female. HCW jobs were categorized into five groups according to those with direct patient care,

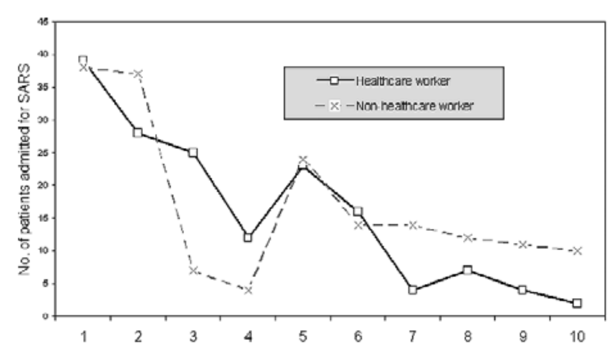

Figure. Number of patients with severe acute respiratory syndrome (SARS) admitted to Prince of Wales Hospital during the first 10 weeks of the SARS outbreak. A total of 160 healthcare workers and 171 non-healthcare workers were admitted; a second peak of admission occurred the 5th week after the outbreak started.

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namely: doctors and nurses, 28% (188); healthcare and general service assistants, 15% (104); and allied health workers, including physiotherapists, occupational therapists, and x-ray technicians, 6% (43). The remainder of staff, who did not have direct patient care, included the ancillary staff, 35% (235); pathology laboratory staff, 14% (95); and others, 1% (9 HCWs). Altogether, 43% of the HCWs reported having known direct contact with patients with SARS or their body fluids, secretions, or excretions. An additional proportion of HCWs might have had contact with patients who subsequently were confirmed to have had SARS, unknown to the HCWs. A total of 36% of the staff worked in or visited adult medical or pediatric wards with SARS patients—30% in the accident and emergency unit and 9% in the intensive-care unit—all areas at high risk for SARS within the hospital during the outbreak. Of the 674 HCWs, 353 (52%) reported mild, self-limiting illnesses during the period between the times when the first and second blood samples were collected (Table). None of the 674 HCWs was shown to have IgG antibody to SARS CoV.

Table. Symptoms reported by healthcare workers without SARS-CoV infection[a]

Symptom[b]	No. (%) of healthcare workers N = 353
Headache	194 (55.0)
Sore throat	174 (49.3)
Cough	140 (39.7)
Coryza	139 (39.4)
Sputum	87 (24.6)
Myalgia	83 (23.5)
Diarrhea	80 (22.7)
Dizziness	75 (21.2)
Chills/rigors	69 (19.5)
Fever	68 (19.3)

[a]SARS-CoV, severe acute respiratory syndrome–associated coronavirus.
[b]All symptoms reported were mild, self-limiting, and lasted for 1 to 2 days.

The current global outbreak of SARS is associated with a novel coronavirus, SARS-CoV, which is phylogenetically distinct from other known members of the virus family (*Coronaviridae*) and genus (*Coronavirus*) (6–8). The full clinical spectrum of this novel infection in humans has not yet been defined. Among the 674 HCWs that we examined, none showed evidence of seroconversion to SARS-CoV.

It is possible that a proportion of our study participants might not have actually been exposed to SARS-CoV. Although these participants were working in our hospital when a large number of patients with SARS were staying there, vigilant infection-control measures had been in place since the outbreak was recognized (9). All staff working in high-risk areas were required to wear a mask, gloves, eye goggles, and protective clothing. These measures have been shown to reduce the risk for infection (10).

The results of this study show that our SARS clinic successfully identified all staff with SARS-CoV infections. Alternatively, our data suggest that asymptomatic or mild forms of SARS-CoV are rare at the current point to which the virus has evolved. From the virologic viewpoint, this finding indicates that the novel coronavirus has not yet adapted to transmit among humans through asymptomatically infected hosts. This finding has important public health implications, as the level of immunity towards SARS-CoV could be very low even in members of communities that had had a large outbreak of SARS. If this is the case, a large proportion of the population remains susceptible, and another major outbreak may occur when the virus is introduced by highly infectious sources.

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Fluoroquinolone Resistance Linked to GyrA, GyrB, and ParC Mutations in *Salmonella enterica* Typhimurium Isolates in Humans

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We report two cases of infection with clonally unrelated, high-level ciprofloxacin-resistant, β-lactamase–producing strains of *Salmonella enterica* Typhimurium. Resistance was caused by four topoisomerase mutations, in GyrA, GyrB, and ParC and increased drug efflux. Ciprofloxacin treatment failed in one case. In the second case, reduced susceptibility to third-generation cephalosporins occurred after initial treatment with these drugs and may explain the treatment failure with ceftriaxone.

Ciprofloxacin, a member of the large and widely used fluoroquinolone group of antimicrobial drugs, is considered the empirical treatment of choice of gastrointestinal infections in adults. The fluoroquinolones have been licensed for use in humans as well as in food-producing animals. This use has raised a worldwide debate on the selection of fluoroquinolone-resistant bacteria in, and the possible circulation between, the different ecosystems concerned (1). While resistance to these drugs occurred quickly in *Escherichia coli* and several other enterobacteriaceae, high-level resistance to ciprofloxacin (HLRC) has been found exceptionally in salmonellae (2–5). The low prevalence of salmonellae with HLRC has been ascribed to counter selection in the environment (6), an observation corroborated by the difficulty in selecting HLRC mutants in vitro. This type of resistance generally involves multiple mutations in the genes encoding the quinolone target enzymes, gyrase and topoisomerase IV (2), and mutations in regulatory systems (*marORAB* [7]) or *soxRS* [8]) or drug efflux systems (AcrAB) (9). We report two cases of infection caused by multiple-resistant *Salmonella enterica* Typhimurium with HLRC in which initial therapy failed and present data on the fluoroquinolone resistance mechanism.

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The Study

Case 1

A 74-year-old man with an aorto-femoral bypass and chronic prostatitis became febrile and was treated empirically with ciprofloxacin. After 2 weeks of persistent fever, he was hospitalized. Pyelonephritis was diagnosed and endocarditis suspected. Urinalysis and blood culture both led to the isolation of a strain of *S.* Typhimurium (STmA) resistant to ciprofloxacin, amoxicillin, tetracycline, chloramphenicol, and sulfonamide. Treatment with cefotaxime was started, the fever subsided, and the patient recovered and was discharged. He did not go for follow-up consultations.

Case 2

A 3-month-old boy (body weight 6 kg) arrived at the hospital with severe diarrhea and fever. A stool culture indicated a strain of *S.* Typhimurium (STmB1) resistant to ciprofloxacin, amoxicillin, tetracycline, trimethoprime, chloramphenicol, streptomycin, spectinomycin, and gentamicin. Treatment was initiated with ceftriaxone IV (250 mg/day) and maintained for 7 days. Signs and symptoms improved for a few days, but fever relapsed after 2 weeks. A second stool culture indicated *S.* Typhimurium strain STmB2, with the resistance phenotype of the original isolate. A second treatment with the same antimicrobial drug IV (300 mg/day) was given for 6 days, again with apparent initial success. However, fever and diarrhea reoccurred after 3 weeks (isolation of strain STmB3 from stool sample). All symptoms disappeared after oral treatment with cefpodoxime (96 mg/day/7 days). The search for carriers within the family was positive in one, with isolation of strain STmC in a 1-year-old sibling who showed no clinical signs of infection.

All STm strains were analyzed for their lysotype, pulsed-field gel electrophoretic (PFGE) pattern, and resistance phenotype and genotype, as previously described (10). Strain STmA was phage nonsusceptible; all STmB and STmC strains were of lysotype 12. The last two had identical PFGE profiles after digestion of genomic DNA with *Xba*I, patterns that were clearly different from that of STmA (5 of 12 fragments were in common). Polymerase chain reaction (PCR) amplification indicated β-lactamase genes in these strains of type TEM in STmA and of type TEM and OXA-1 in the STmB and STmC strains. MICs of the antimicrobial drugs shown in the Table were determined for the five clinical strains and the reference strain

Table. Antimicrobial drug susceptibilities of the *Salmonella enterica* Typhimurium isolates

Isolate	MIC (mg/L)[a]			
	NOR	CIP[b]	CTM	CTX
NCTC 12416	0.06	0.016	0.06	0.125
A	32	16	0.06	0.06
B1	32	32	0.25	0.125
B2	32	32	1	0.25
B3	32	32	1	0.25
C	32	32	0.25	0.125

[a]NOR, norfloxacin; CIP, ciprofloxacin; CTM, cefotaxime; CTX, ceftriaxone.
[b]MICs of moxifloxacin were identical to those of CIP.

S. Typhimurium NCTC 12416 (phage type LT2), on Mueller-Hinton medium containing serially twofold diluted antimicrobial drugs. MICs of ethidium bromide and of ciprofloxacin, cefotaxime, and ceftriaxone in the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France) (11) at concentrations of 50 mg/L were also determined. All clinical strains were similarly high-level resistant to fluoroquinolones. Isolate STmA, producing the TEM β-lactamase, was more susceptible to cefotaxime and ceftriaxone than the STmB and STmC isolates producing in addition OXA-1, and the post-treatment STm strains B2 and B3 were two- to fourfold less susceptible to the third-generation cephalosporins than STmB1 (Table). In the presence of Phe-Arg-β-naphthylamide at 50 mg/L, a concentration at which no inhibition of growth was observed in the controls, MICs of ciprofloxacin were reduced fourfold and those of cefotaxime and ceftriaxone were reduced twofold. All STm isolates showed strongly reduced susceptibility to ethidium bromide (MIC $\geq$ 1,000 mg/L as opposed to $\leq$100 mg/L for the reference strain).

To identify mutations responsible for HLRC, the quinolone resistance determining regions (QRDR) of DNA gyrase and topoisomerase IV were determined after nucleotide sequencing of the corresponding QRDR fragments which were PCR-amplified with the respective pairs of primers: 5′CTGAAGCCGGTACACCGTCG and 5′TCGGCCATCAGTTCGTGGGC for *gyrA*; 5′TTATCGATGCTGCGCGTGCC and 5′TCGCCGCTTTCAGGGCGTTC for *gyrB*; 5′CGCCTACTTAAACTACTCCA and 5′ATCAGCGTAATCGCCGCTTT for *parC*; and 5′GACC-GAGCTGTTCCTTGTGG and 5′GCGTAACTGCATCGGGTTCA for *parE*. The QRDRs of the topoisomerases of the five strains had identical mutations in GyrA (Ser83Phe and Asp87Asn) and in GyrB (Ser464Phe), while distinct mutations were observed in ParC (Glu84Lys in strain STmA and Ser80Arg in all STmB and STmC strains). The recently described quinolone resistance-conferring gene *qnr* was absent from all STm strains as tested by PCR with the corresponding specific primers (12). Also, nucleotide sequencing did not show any mutation in the PCR-amplified *marORA* locus.

Electrophoretic (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis of outer membrane proteins indicated the loss of one of them, possibly a porin (data not shown), in the post-treatment isolates STmB2 and STmB3. These changes did not influence the levels of fluoroquinolone resistance but were associated with reduced susceptibility to cefoxitin (not shown) and cefotaxime (fourfold) and to ceftriaxone (twofold) (Table).

Conclusions

The two cases reported here show that clonally unrelated HLRC strains of *S.* Typhimurium, which may cause severe infections, are present in the community. In both cases initial treatment failed, respectively, with a fluoroquinolone and a third-generation cephalosporin. In the first case, the failure may be attributable to the multiple topoisomerase mutations (possibly in conjunction with increased drug efflux), although treatment failures have been reported in cases of lower levels of resistance to ciprofloxacin (13). In the second case, the reason for the initial treatment failure with ceftriaxone is less obvious. Strain STmB1 had the potential to decrease its susceptibility to third-generation cephalosporins under treatment, possibly related to the loss of an outer membrane protein**.** The high-level resistance to ethidium bromide and the increased susceptibility of the strains to ciprofloxacin in the presence of Phe-Arg-β-naphthylamide were strongly suggestive of an activated drug efflux system, such as AcrAB, the substrates of which also include cephalosporins with lipophilic side chains (2,14). The growth rates of the strains were the same as that of the reference strain, an observation which is at variance with that made with experimental fluoroquinolone-resistant mutants selected in animals (6) and which might imply the existence of additional compensatory mutants in the human STm isolates.

Low-level ciprofloxacin resistance in salmonellae has been observed occasionally in the environment in France (15) and elsewhere (16). Since we did not have a pretreatment isolate at our disposal, we are not certain whether any of the mutations resulting in the high-level fluoroquinolone resistance observed in strain STmA were selected under treatment. On the other hand, HLCR strain STmB may have been acquired as such from the environment or through the food chain, since neither the patient nor his sibling had ever been treated with fluoroquinolones, although no salmonellae with this phenotype have been observed to date from environmental or animal sources in France. In fact, to our knowledge, quadruple mutations affecting three topoisomerase subunits have never been reported before in salmonellae.

The unwelcome occurrence of fluoroquinolone-resistant salmonellae accumulating multiple mechanisms of resistance could compromise standard therapy of infections attributable to such pathogens. For children, an initial high-dose regimen with commonly used third-generation cephalosporins, or with oral cephalosporins, may therefore be indicated for the treatment of infections caused by *S.* Typhimurium strains such as STmB1.

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Cowpox with Severe Generalized Eruption, Finland

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Cowpox with a severe, generalized eruption was diagnosed in an atopic 4-year-old girl by electron microscopy, virus isolation, polymerase chain reaction, and immunoglobulin (Ig) M and low-avidity IgG antibodies. The hemagglutinin gene of the isolate clustered with a Russian cowpox virus strain, and more distantly, with other cowpox and vaccinia virus strains. The patient's dog had orthopoxvirus-specific antibodies, indicating a possible transmission route. In Finnish wild rodents, orthopoxvirus seroprevalences were 0%–92%, in humans the seroprevalence was 100% in the age group >50, decreasing towards younger age groups.

Cowpox is a zoonotic dermatitis affecting, despite its name, mainly cats and humans. The disease is caused by cowpox virus, a close relative to vaccinia, smallpox (variola), and monkeypox viruses within the *Orthopoxvirus* genus (1). The relationship between cowpox and vaccinia viruses has been unclear since Edward Jenner used a virus isolate from cows for smallpox vaccination (2,3). Orthopoxviruses, comprising a genus in the family *Poxviridae,* are large, brick-shaped viruses with a 200-kbp DNA genome, and they replicate in the cytoplasm (4). Because immunity to orthopoxviruses is cross-reactive, smallpox vaccination might have suppressed cowpox virus infections in the human population. Cowpox virus is not highly infective for humans and usually produces a localized lesion mainly on fingers, hands, or face (5). In immunocompromised persons, however, the disease may lead to death (6). The virus infects through skin abrasions, resulting in successive lesions of macular, papular, vesicular, pustular, ulceral, and eschar stages for 2 weeks. Systemic symptoms are also common (5). The reservoir hosts of cowpox are wild rodents (7); wild rodents may transmit the virus to humans through cats (5) or other pets that roam outside. Direct transmission from a rodent to a girl has been recently described (8). Both cowpox and monkeypox, which was recently transmitted to the United States by transport of animals indigenous to Africa (9), are actually orthopoxviruses of their reservoir rodents and are not well adapted to interhuman spread (1). The misleading nomenclature is based on the hosts from which they were first identified (1). Cowpox virus infections have been detected in Europe and central and northern Asia (1). Nongeneralized infections in children have been previously characterized and diagnosed by electron microscopy (10), virus isolation, polymerase chain reaction (PCR), and restriction enzyme analysis (10,11).

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The Study

A 4-year-old girl from a small farm in eastern Finland was hospitalized in September 2000 because of umbilicated vesicopapules, which developed over the previous 5 days (Figure 1), and unresponsiveness to cephalexin. She had a past history of moderate atopic dermatitis. Animals at her home farm included a horse, three dogs, and a rabbit, but she had no contact with cats because of allergy. On examination, most lesions were located on her swollen red extremities, a few were found on the side of her body, and 3-mm lesions were found on the face and vulva. All lesions were at the same stage of development. She was febrile with 38°C temperature and feeling unwell. A biopsy sample from a papule was sent to a virology laboratory, where orthopoxvirus particles 230 × 300 nm in size were demonstrated by electron microscopy with negative staining.

The girl was treated in isolation at the hospital with chlorhexidine washings and wound dressings with fusidic acid. Intravenous dicloxacillin was administered to prevent secondary bacterial infections. On day 12, skin lesions progressed to deep-seated, hard, black eschars. At the same time the patient's general condition improved. Two months later, all lesions were healed with scars.

A homogenized biopsy sample was used to infect Vero cells, where a cytopathic effect typical of orthopoxviruses

Figure 1. Cowpox lesions on patient's forearm on day 7 after onset of illness.

was seen after 2 days. The isolate, designated CPXV/FIN/T2000, was shown to contain orthopoxvirus by electron microscopy, and DNA samples from both the infected cells and the original biopsy specimen were PCR-positive for orthopoxvirus thymidine kinase gene (12). The hemagglutinin (HA) gene of the isolate was amplified (13), sequenced (948 nucleotides; accession no. AY366477), and compared to other orthopoxviruses. The CPXV/FIN/T2000 strain differed 3% to 4% at the nucleotide level from cowpox virus strains available in GenBank. The sequences were further subjected to phylogenetic analysis: they were aligned by using Clustal X with Gonnet protein matrix and analyzed by using the maximum likelihood phylogenetic software TREE-PUZZLE 5.0, applying the Hasegawa model of substitution and performing 25,000 steps. The HA sequence of CPXV/FIN/T2000 formed a separate clade with cowpox virus strain GRI-90 (Figure 2), isolated originally from another 4-year-old girl, who contracted cowpox after playing with a mole near Moscow (14). The reference cowpox virus strain Brighton grouped with camelpox and variola viruses instead of other cowpox or vaccinia viruses. However, because of the high homology of HA genes, this finding should be interpreted with caution. The remaining cowpox virus strains clustered together with vaccinia viruses; this clustering may, in some cases, be explained by origin from vaccinia virus strains that had escaped to nature (13). This finding seems not to be the case in Finland, since the HA nucleotide sequence of vaccinia virus used in Finland differed 4% both from the strain CPXV/FIN/T2000 and another Finnish cowpox virus isolate from 1989 with an identical HA sequence (data not shown). Different alignment parameters and the neighbor-joining method produced the same results with high support values. Scattered phylogenetic distribution of cowpox virus strains is supported by data presented by other researchers and may reflect an ancestral role of cowpox viruses within the *Orthopoxvirus* genus; some strains cluster with vaccinia viruses and others (including the reference Brighton strain) with variola virus (1).

An immunofluorescence assay (IFA) to measure specific immunoglobulin (Ig) G, IgM, and avidity of IgG antibodies (15) was established on acetone-fixed, CPXV/FIN/T2000-strain-infected Vero cells. The patient's serum had a high IgM-antibody titer at admission and low avidity of specific IgG; after 60 days, the IgM level was low and the IgG avidity high (Table 1).

The serum samples from the patient's pets were collected later and tested with IFA. A hunting dog had antibodies with a titer of 320; another dog had a titer of 20. Thus, a dog might have transmitted the infection from a wild rodent to the patient, although cats are thought to be the main source of human infection (5).

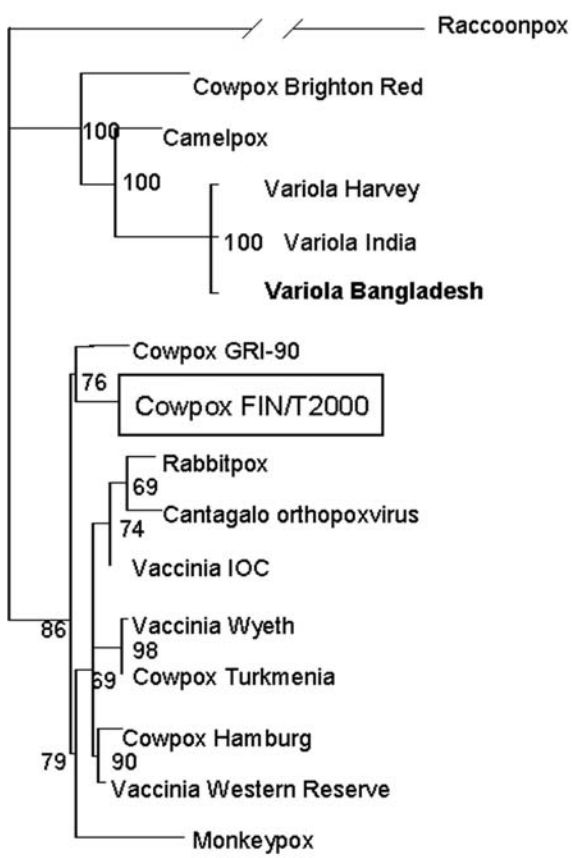

Figure 2. Phylogenetic tree of selected orthopoxvirus hemagglutinin genes based on Clustal X alignment and the maximum likelihood method TreePuzzle. Virus sequences used for the analysis were raccoonpox as an outgroup (GenBank accession no. M94169); cowpoxvirus strains Brighton Red (AF482758), FIN/T2000 (AY366477), GRI-90 (Z99047), Hamburg (Z99050), and Turkmenia (Z99048); vaccinia virus strains IOC (AF229248), Western Reserve (M93956), and Wyeth (Z99051); variola virus strains Bangladesh (L22579), Harvey (X65516) and India (X69198); camelpox virus (AF438165); Cantagalo orthopoxvirus (AF229247); monkeypox virus (Z99049); and rabbitpox virus (Z99049).

Table 1. Orthopoxvirus antibodies in sera of the patient and her pets[a]

Characteristic	Titer		
	IgM	IgG	IgG avidity (%)
Patient day 7	1280	2560	1.5
Patient day 14	640	2560	3.1
Patient day 60	10	640	25
Old-immunity controls (>50 y)	<10	80–640	50–100
Negative controls	<10	<10	—
Dog A	—	<20	—
Dog B	—	320	50
Dog C	—	20	50
Horse	—	<20	—
Rabbit	—	<20	—

[a]Ig, immunoglobulin.

We further studied orthopoxvirus antibodies by IFA in Finnish fauna and humans. The seroprevalence rates for cats and horses were 3.9% and 1.6%, respectively; the rates for wild rodents (when either serum or lung or heart extracts was used) were 0% to 92%, depending on the trapping time and the location (Table 2). In Värtsilä, a rural district in eastern Finland near where the patient lives, antibodies were found in 1 (2.8%) of 36 rodents. However, variation of seroprevalence rates in rodents is also influenced by population dynamics: low prevalences are often found in the increase phase and high prevalences in the peak phase of rodent population fluctuations. The positive rodents were mainly bank voles *(Clethrionomys glareolus*). The two seropositive horses were from a region near where the patient lives in eastern Finland, and the three seropositive cats were free-roaming and found in southern Finland.

Table 2. Orthopoxvirus antibodies in cat, horse, and wild rodent panels, Finland

Panel	n	Positive	Prevalence (%)
Cats	77	3	3.9
Horses	127	2	1.6
Wild rodents			
Southern Finland: Evo	36	33	91.7
Eastern Finland: Värtsilä	36	1	2.8
Western and central Finland:			
Several localities	436	0	0
Lapland: several localities	394	7	1.8 (0.4–15.2)
Wild rodents altogether	902	41	4.5

In addition, seroprevalence rates of 50% (7/14) and 1.4% (1/73) have been found in foxes and lynxes, respectively, from a limited geographic area in Finland (16).

Sera collected at a Finnish Veterinary meeting in 2001 showed that every person >50 years had orthopoxvirus antibodies, as measured by IFA. The seroprevalence decreased gradually for younger age groups (Table 3), reflecting the gradual cessation of smallpox vaccination (the last vaccinations in Finland occurred in 1977). The average population might have lower seroprevalence rates than veterinarians because of veterinarians' frequent contact with animals that may harbor orthopoxvirus. Consequently, younger age groups are more susceptible to smallpox and cowpox virus infections.

Although cowpox virus infection usually causes a single, painful, ulcerated vesicopustule and local lymphadenopathy, immunocompromised patients and children, especially those with atopic eczema, are susceptible to a generalized, even lethal, smallpoxlike infection (5,6). An early diagnosis and prompt recognition of the virus are essential for treating and differentiating cowpox, from other orthopoxvirus and herpesvirus infections, especially in severe cases.

Table 3. Orthopoxvirus antibodies in humans (veterinarians), Finland

Humans (y)	n	Positive	Prevalence (%)
≤25	19	2	10.5
26–30	23	4	17.4
31–50	78	46	59.0
≥51	18	18	100.0

Conclusions

Cowpox virus (orthopoxvirus) infection was diagnosed by electron microscopy, PCR, virus isolation, and serologic testing (positive IgM or low avidity of IgG antibodies). Cowpox virus strains show considerable genetic variations with different positioning in the orthopoxvirus phylogenetic tree. Circulation of cowpox virus in wild and domestic animals, together with decreased immunity in humans, may lead to increased occurrence of human cowpox, especially in atopic and immunocompromised persons who are at risk for generalized infection. The described case further suggests atopy to be a contraindication to smallpox vaccination.

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Pulmonary Tuberculosis due to *Mycobacterium bovis* subsp. *caprae* in Captive Siberian Tiger

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We report the first case of pulmonary tuberculosis caused by *Mycobacterium bovis* subsp. *caprae* in a captive Siberian tiger, an endangered feline. The pathogen was isolated from a tracheal aspirate obtained by bronchoscopy. This procedure provided a reliable in vivo diagnostic method in conjunction with conventional and molecular tests for the detection of mycobacteria.

Mycobacterium bovis, a member of the *M. tuberculosis* complex (MTBC), can cause tuberculosis in a wide range of domestic and wild animals and also in humans (1,2). Routine differentiation of *M. bovis* is based on a number of phenotypic characteristics and biochemical tests (2). *M. bovis* shows dysgonic growth on Löwenstein-Jensen (LJ) medium and has been described as negative for nitrate reduction and niacin accumulation (2). As a further criterion for the differentiation of *M. bovis*, intrinsic resistance to pyrazinamide (PZA) has been described (2). However, more recently, PZA-susceptible strains of *M. bovis* were found in Spain and Germany; these strains were also characterized by specific molecular techniques (3–5). As a consequence, *M. bovis* was split into two subspecies: *M. bovis* subsp. *bovis*, which showed resistance to PZA, and *M. bovis* subsp. *caprae*, which was sensitive to PZA (6,7). *M. bovis* subsp. *caprae* was initially isolated from sheep and goats in Spain (3,4,7); however, further studies confirm its infectivity in humans, cattle, and red deer (6,8). We report the unusual case of a *M. bovis* subsp. *caprae* infection in a captive Siberian tiger.

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Case Report

An 8-year-old male Siberian tiger at the Budapest Zoological and Botanical Garden had episodes of coughing in October 2001. Because the coughing did not stop in 6 to 7 days, an expectorant (Bisolvon; Boehringer Ingelheim Vetmed Gmbh., Ingelheim am Rhein, Germany) was given for 10 days. His condition showed a temporary improvement; however, after a few weeks, the animal started coughing again, and his appetite decreased. Amoxicillin plus clavulanic acid (Amoksiklav; Lek Animal Health, Ljubljana, Slovenia) and ketoprophen (Ketofen, Merial, Lyon, France) therapy was given for 7 days. The tiger's condition did not show any notable improvement. In addition, in May 2002, the animal's respiratory rate became elevated, he became dyspneic and emaciated, and his daily activity substantially decreased. Further antibacterial treatment was administered (cefatroxil, Cefa-cure; Intervet, Boxmeer, the Netherlands) during that month without clinical effect. At that point, the animal was anesthetized, and tracheoscopy was performed with a flexible 56-cm bronchoscope (Olympus B3R; Tokyo, Japan (Figure 1). The examination found a large amount of purulent mucus in the trachea. Therefore, several tracheal washings were taken for microbiologic tests by using a commercially available tracheal suction set (Medinorm Medizintechnik GmbH, Quierschied, Germany (Figure 1). A chest radiograph showed a severe and extensive bronchointerstitial pattern with cavernous lesions in both lungs.

Nine days after the specimens were taken, cultures for mycobacteria showed growth in the broth-based MGIT 960 system (Becton-Dickinson Microbiology Systems, Sparks, MD). The acid-fast organism that was isolated was identified as MTBC by the AccuProbe TB assay (Gen-Probe Inc., San Diego, CA).

Since the tiger had stopped eating and his condition had dramatically deteriorated, the animal was euthanized and

Figure 1. Obtaining a tracheal washing of the Siberian tiger by bronchoscopy.

necropsy was performed. Hematoxylin and eosin–stained histologic sections of the lung segments showed an extensive multifocal infiltration of lymphocytes, histiocytes, and some scattered multinuclear giant cells within the framework of proliferated connective tissue and collagen fibers of the cavernous lesions. Ziehl-Neelsen staining showed an intracellular accumulation of acid-fast bacteria in several alveolar macrophages and epithelioid cells.

The keepers of the tiger also underwent pulmonary radiographs and tuberculin skin testing. Their skin test results were negative, and clinical or radiologic signs of tuberculosis were not detected.

Characterization of MTBC Isolate

Colony morphology of the isolated MTBC strain showed dysgonic growth on LJ medium and microaerophilic growth on Lebek medium. The strain was susceptible to PZA (100 µg/mL) and thiophen-2-carboxylic acid hydrazide (TCH; 1 µg/mL) and negative for niacin accumulation and nitrate reduction (9–11).

The genome of the isolate was analyzed for specific mutations in the *pncA*, *oxyR*, and *gyrB* genes by automated DNA sequencing, polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique, and spoligotyping as described previously (5,12–16). Susceptibility of the isolate to PZA was linked with a wild-type *pncA* sequence. In addition, the isolate contained the *M. bovis*–specific G-to-A mutation at position 285 in the *oxyR* gene and the G-to-A mutation at position 756 in the *gyrB* gene. However, spoligotyping showed a pattern with the absence of spacer sequences 39–43 and 3–16 (Figure 2), and T to G mutation at position 1,311 in the *gyrB* gene, characteristic of *M. bovis* subsp. *caprae,* could also be detected by DNA sequencing. On the basis of these phenotypic and genetic characteristics, the strain was identified as *M. bovis* subsp. *caprae* (3,6,7,17).

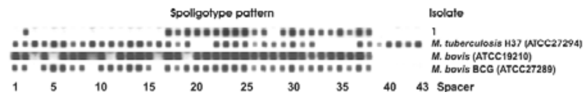

Figure 2. Spoligotype patterns of the isolate obtained from the Siberian tiger: lane 1; spacer sequences 3–16 and 39–43 are absent); lane 2, control strain *Mycobacterium tuberculosis* H37Rv ATCC 27294; lane 3, control strain *M. bovis* ATCC 19210; lane 4, M. bovis BCG ATCC 27289.

Conclusions

MTBC comprises these closely related organisms: *M. tuberculosis; M. africanum; M. bovis*; the vaccine strain, *M. bovis* bacillus Calmette-Guérin; and three rarely seen members, *M. microti*, *M. canettii,* and the recently described seal bacillus, *M. pinnipedii* (17–20). Differentiation within MTBC is necessary for individual patient treatment (i.e., inclusion or exclusion of PZA) and for epidemiologic purposes, especially in areas of the world where tuberculosis has reached epidemic proportions or wherever the transmission of *M. bovis* between animals, animal products, and humans is a problem (10).

The host range of *M. bovis* is wide, including many animal species and humans. Carnivores such as large felines may acquire the infection through the alimentary tract by eating infected meat (4). Reports of tuberculosis in large captive or free-living felines are not common (21–26), however.

To our knowledge, this case is the first in which tuberculosis attributable to *M. bovis* subsp. *caprae* was diagnosed in a large feline. The rapid and accurate in vivo diagnosis of tuberculosis is indispensable in endangered captive animals such as the Siberian tiger, not only because of the declining population of this species but also to prevent the transmission of the disease to other animals. Although nasal or throat swabs are used most often, we found tracheal washing by bronchoscopy was easy to perform, rapid, and more adequate than swabs (provided a larger sample volume from the lower airways) for obtaining clinical specimen for mycobacterial or other microbiologic tests.

The rapid diagnosis of tuberculosis is essential for adequate antituberculosis treatment to be started as early as possible. The effectiveness of antituberculosis therapy in felines is controversial (27). However, when an endangered animal is involved, early diagnosis of the disease might help control it in time to save the animal, especially with the help of a rapid in vivo diagnostic method such as tracheal washing through bronchoscopy. Tracheal washing can also be the method of choice to bacteriologically monitor the efficacy of therapy. In this case, the poor appetite and condition of the animal did not allow survival long enough for delivery of antituberculosis treatment. The source of infection could not be conclusively identified retrospectively; infected goat meat (a usual diet of the animal) is a likely possibility because the tuberculosis-related control measures are not as strict with goats as with cattle in Hungary (annual tuberculin skin testing of goats is not mandatory, for example) (28).

This report indicates that routine differentiation within the MTBC is indispensable for understanding the epidemiology of tuberculosis and for determining the prevalence, transmission, and clinical importance of the different members of the complex.

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novel diagnostic methods for detecting mycobacterial infections and identifying mycobacteria.

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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.

Dengue-1 Virus Isolation during First Dengue Fever Outbreak on Easter Island, Chile

Cecilia Perret,* Katia Abarca,* Jimena Ovalle,* Pablo Ferrer,* Paula Godoy,* Andrea Olea,† Ximena Aguilera,† and Marcela Ferrés*

Dengue virus was detected for the first time in Chile, in an outbreak of dengue fever on Easter Island. The virus was isolated in tissue culture and characterized by reverse transcription–polymerase chain reaction as being dengue type 1.

Dengue fever (DF) is a common viral disease of the tropics. Only a few countries in the Americas, including Chile, have not reported cases of this disease. Distribution of dengue virus in the Americas has increased since 1970, when efforts to eradicate the vector (*Aedes aegypti*) waned, particularly in Central America and the Amazon region. Not only has the number of DF cases on the continent (1) increased but all four types of dengue virus have also been introduced. Consequently, the number of dengue hemorrhagic fever cases (DHF) has risen because secondary infections are now common in populations in which multiple dengue serotypes are circulating.

At the beginning of 20th century, *A. aegypti* existed in northern Chile, where the climate is suitable for the mosquito to breed, but it was eradicated in 1945 (2). Since then, no evidence of reintroduction of the mosquito was observed by entomologic surveillance. However, by the end of 2000, the presence of the mosquito was confirmed on Easter Island (3), which is located in the Pacific Ocean 3,800 km off the coast of Chile. All of the island's 3,860 inhabitants live in one village, Hanga Roa, on the western coast. At that time, 70% of the houses of this village were infested by *A. aegypti,* according to studies performed by the Epidemiological Unit of the Ministry of Health (4). Devices to catch mosquito larvae were installed in a sampling of houses, in the rural sectors, and near the three volcano lakes. The larvae were found in the entire urban sector, in some sections of the rural areas (Vaitea y Tahai), and in none of the volcano lakes (5). Educational campaigns and control efforts (insecticides and reduction of container breeding sites) were carried out to decrease mosquito infestation. During the 2002 dengue outbreak, an average of 5% of the sampled houses were infested.

Before the outbreak on Easter Island, 15 cases of DF had been diagnosed in continental Chile in 2000 and 2001 and serologically confirmed in our laboratory. Dengue was acquired for all case-patients during when traveling within the American continent.

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The Study

The index case-patient, a 21-year-old Chilean woman, had been living on Easter Island for 2 months and had not traveled. She had a high temperature (39°C), myalgias, arthralgias, headache, and a maculopapular rash for 7 days. Laboratory analysis of a blood sample indicated low leukocyte and platelet counts. While still febrile, she traveled to Santiago, the capital of Chile, and was admitted to a private hospital; DF was suspected. On March 13, 2002, DF was confirmed by an in-house dengue immunoglobulin (Ig) M enzyme-linked immunosorbent assay (ELISA) in our laboratory. This case of DF was the first acquired in Chile.

The Ministry of Health organized an outbreak investigation team. As part of the study and with the goal of recovering and identifying the virus, blood samples were taken from 16 febrile patients who were assessed and satisfied the clinical definition of suspected dengue case made by the Ministry of Health. The samples were brought to our laboratory, and plasma was used for viral culture and for reverse transcription–polymerase chain reaction (RT-PCR). Serologic testing was not performed on these samples.

Viral culture was attempted from 15 acute-phase plasmas. Plastic flasks (T-25) seeded with Vero cells were injected with 200 µL of plasma diluted 1:5 with medium 199, 2% fetal bovine serum, gentamycin 50 µg/mL. After 1 hour of absorption at 37°C, cultures were incubated 10 days at the same temperature and observed once a day for cytopathic effect (CPE). Cells were harvested for indirect immunofluorescence antibody testing (IFAT) after CPE was first observed (as early as day 5 in some of the cultures and on day 10 of incubation in all the other samples). Initially, IFAT was performed with polyclonal antisera reactive with all serotypes (D1–D4); then samples with positive results were stained with monoclonal antibodies specific for each subtype to identify dengue serotypes.

A nested RT-PCR developed by Lanciotti (6) was used to analyze plasma and viral culture supernatants from 15 febrile patients. Samples (200 µL) were taken, and RNA was extracted with Trizol (Gibco BRL, Life Technologies, Rockville, MD). RNA (5 µL) was reverse transcripted and cDNA amplified with primers D1 and D2, SuperScript II, *Taq* polymerase (Gibco) in a single reaction vessel with 50 µL final volume. The thermocycler was programmed to incubate for 1 h at 42°C and then 35 cycles at 94°C, 55°C, and 72°C. The second step used 10 µL of diluted 1:100 dengue cDNA from the first reaction and contained primers to amplify the four dengue serotypes (TS1–TS4, plus D1). The results had bands of different sizes, depending on the serotype (DENV-1 482 bp, DENV-2 119 bp, DENV-3 290 bp and DENV-4 392 bp) after 20 cycles at the same temperatures as the first reaction.

Dengue virus was isolated from 13 of 15 acute-phase plasmas by viral culture. One of the negative plasma samples was from a patient who was febrile for 5 days. The isolated dengue virus was identified as DENV-1 serotype by IFAT by using monoclonal antibodies in slides prepared from the viral cultures (Figure 1) and by RT-PCR obtaining a band of 482 bp (Figure 2).

When Lanciotti primers design was used, RT-PCR amplified virus RNA from the 13 positive cell culture supernatants but from none of the acute-phase plasmas. To improve sensitivity, the primer TS1 was modified, decreasing the Cs and Gs at the 3′ end. The new primer was located in the genome position 575–595, instead of 568–586 (7), amplifying a DNA product of 491 bp. Using this new TS1 primer, we could amplify dengue-1 RNA in 8 of 15 plasmas; none of the negative cultures plasmas was positive by PCR.

In addition to the virologic study, a serum sample was taken from 423 asymptomatic convalescent patients who recalled being febrile during the last 2 months. These samples were tested for dengue IgM by ELISA at the National Reference Laboratory of the Ministry of Health; 176 were IgM positive.

According to the epidemiologic results, the outbreak was from January to May 2002, and 636 cases of DF were diagnosed. A total of 460 cases were diagnosed by epidemiologic nexus, satisfying the case definition, and 176 were confirmed by IgM serologic testing. Therefore, the incidence rate of the disease was 16.6% (4). No cases of DHF were diagnosed.

Conclusions

The isolation of the virus from febrile patients during the outbreak confirmed the first appearance of dengue virus in insular Chile and the fact that the virus causing the epidemic is DENV-1. The identification of the virus has allowed us to presume that the original source of the virus might be tourists from either Brazil or Tahiti. Most of the tourists (45%) visiting Easter Island came from Brazil. A lower proportion came from the Pacific Islands, where the same virus serotype was circulating at the time the out-

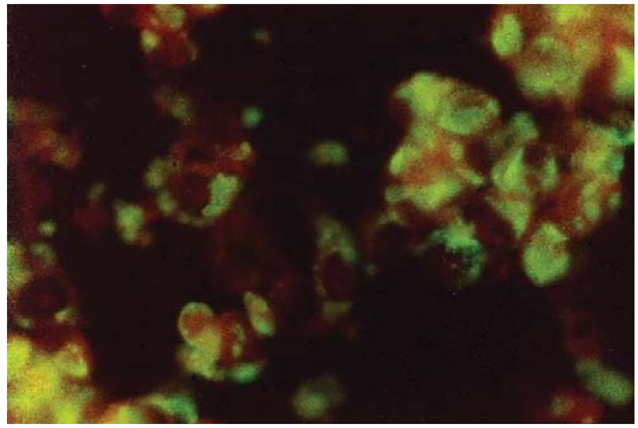

Figure 1. Indirect immunofluorescence antibody testing with monoclonal antibodies identifying dengue-1 virus in tissue culture of Vero cells.

break started. Knowing the serotype is important to keep a strict surveillance of febrile patients and mosquitoes to determine if a different dengue virus serotype is introduced and to determine if cases of DHF are appearing on the island.

Laboratory tests, like serology (IgM and IgG ELISA), and RT-PCR for dengue virus, were already available at our laboratory, whereas viral culture with IFAT for virus identification was quickly developed when the DF outbreak was identified. The further genotyping of the isolated dengue virus will allow us to compare with other DENV-1 viruses circulating in other parts of the world and determine the origin of Easter Island DENV-1.

Because of diagnosis of the first indigenous case of DF in a country where tropical infections are unusual, being

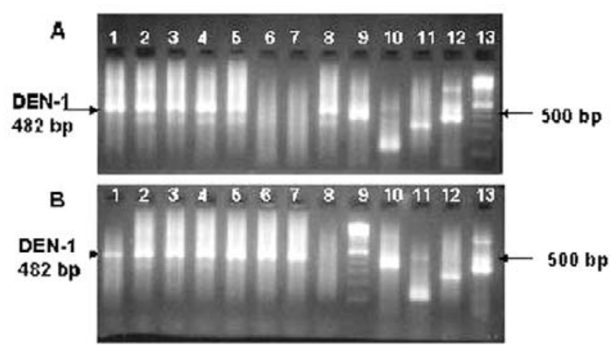

Figure 2. Visualization of reverse transcription–nested polymerase chain reaction product from 15 cultures of supernatant. DENV-1 positive samples are indicated by a 482-bp band. A) Lanes 1–5: positive culture supernatants. Lanes 6–7: negative culture supernatants. Lane 8: positive culture supernatant. Lane 9: positive DENV-1 control. Lane 10: positive DENV-2 control. Lane 11: positive DENV-3 control. Lane 12: positive DENV-4 control. Lane 13: 100-bp DNA ladder. B) Lanes 1–7: positive culture supernatants. Lane 8: negative control. Lane 9: 100-bp DNA ladder. Lane 10: positive DENV-1 control. Lane 11: positive DENV-2 control. Lane 12: positive DENV-3 control. Lane 13: positive DENV-4 control.

able to make a differential diagnosis and having laboratory resources for a variety of emerging infectious diseases are important, particularly for an immunologically naive community, such was the case of Easter Island and the Chilean population.

Acknowledgments

We thank the Easter Island population for providing blood samples, the physicians for their cooperation, the Ministry of Health for allowing us access to the outbreak information data, our laboratory staff for their enthusiasm in quickly developing new methods, Juan Pascale for sending us dengue monoclonal antibodies, and the Naval Medical Research Center Detachment for helping us with the protocols to develop the dengue virus culture and supplying us with the reagents for the in-house dengue enzyme-linked immunosorbent assay.

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Flow Cytometry and T-Cell Response Monitoring after Smallpox Vaccination

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Orthopoxvirus zoonosis or smallpox as result of bioterrorism or biological warfare represents a risk for epidemic spread. By monitoring T-cell responses by flow cytometry, we observed a recall response after recent vaccination against smallpox. When the high similarity between the orthopoxviruses is considered, this rapid assay that uses vaccinia antigens could identify recently exposures.

Although the last natural case of smallpox was reported in Somalia in 1977, this orthopoxvirus remains a source of concern. No evidence exists that smallpox will recur as an endemic disease, but the virus may have been acquired for use in biological warfare or bioterrorist attacks. If one assumes an average of 15 days is needed for infected persons to become infectious, delay in intervention will be costly, increasing the total number of cases (1). Furthermore, the recent outbreak of the severe acute respiratory syndrome coronavirus and the first documented outbreak of monkeypoxvirus in the Western Hemisphere underline the ever-present risk for epidemic extension of zoonosis and raise concerns about the medical and social effect of reemerging orthopoxvirus infection in humans. During the epidemic spread of an emerging pathogen, evaluating exposed persons and containing the infected population should be the first priorities. A local outbreak of orthopoxvirus infection would require rapid and sensitive diagnostics, including novel assays based on host responses.

For intracellular pathogens, the antibody titers and neutralization assays represent routine immunologic tests that provide results after several weeks of infection. The appearance of a detectable antibody titer takes place a few days after the induction of a T-cell response (2). Moreover, antigen-specific T-cell responses could be detected in exposed, but uninfected persons, as shown in those with HIV infection (3). Using a rapid flow cytometric test, we previously showed that monitoring interferon (IFN)-γ production by antigen-pulsed T cells provides a quantitative and functional assessment of HIV- or cytomegalovirus (CMV)-specific CD8(+) and CD4(+) T cells (4–6). This technique requires that whole proteins or selected peptide antigens are added to blood cells, allowing the simultaneous analysis of both major histocompatibility complex class I and II restricted T-cell responses (7). Because smallpox vaccination was recently shown to induce a strong vaccinia virus-specific CD8(+) CTL- and IFN-γ–producing T cells detectable by more cumbersome research laboratory methods (cytotoxic, proliferative, or ELISPOT assays) (8,9), we evaluated the feasibility of an easy, rapid, and sensitive assay to monitor T-cell responses after recent vaccination against smallpox; the assay can potentially be used as a routine diagnostic assay.

The Study

T-cell reactivity was analyzed after recent (<2 years ago) smallpox vaccinations, in long-term vaccinated (>20 years ago) and not vaccinated persons. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by standard density centrifugation (Ficoll-Hypaque, Pharmacia, Uppsala, Sweden). Stimulation was also performed on whole blood samples; however, the assay had reduced sensitivity. We cannot exclude the possibility that whole blood assay sensitivity could be improved by changing protocol conditions (data not shown). PBMC were cultured in complete Roswell Park Memorial Institute 1640 medium, 10% v/v heat-inactivated fetal calf serum, 2 mM L-glutamine, and 10 U/mL penicillin/streptomycin at a concentration of 10^6 cells/mL. Stimulation was performed with 40 µL/mL (total protein content of approximately 1 µg/mL) of vaccinia viral antigen resuspended according to the manufacturer's instructions (Maine Biotechnology Services, Portland, ME), or 2 µg of CMV antigen (Biowhittaker, Walkersville, MD), always in the presence of co-stimulation with both anti-CD28 and CD49d monoclonal antibodies (1 µg/mL, Becton, Dickinson and Company, Franklin Lakes, NJ). We also tested the T-cell response with live vaccinia-infected fibroblast or Vero cells. The response against uninfected antigenic preparations was always above background, reducing the sensitivity of the assay (data not shown); therefore, the commercially available antigens were used in subsequent experiments. Cultures were incubated at 37°C for 1 h, followed by an additional overnight incubation with 10 µg/mL of the secretion inhibitor Brefeldin-A (Sigma-Aldrich Corporation, St. Louis, MO). Cells were washed twice in phosphate-buffered saline, 1% bovine serum albumin, and 0.1% sodium azide, and stained for 15 min at 4°C with monoclonal antibodies specific for cell surface CD antigens (Becton,

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Dickinson and Company). Samples were then fixed in 1% paraformaldehyde for 10 min at 4°C, incubated with Phyco-Erithrin-conjugated mouse-anti-human IFN-γ (Becton, Dickinson and Company), washed twice in phosphate-buffered saline, 1% bovine serum albumin, and 0.1% saponin, and resuspended in FACSFlow before being acquired on FACScalibur (Becton, Dickinson and Company), as previously described (4,6). Controls for nonspecific staining were monitored with isotype-matched monoclonal antibodies (Becton, Dickinson and Company); cells incubated with only anti-CD28 and -CD49d were included in each experiment and nonspecific staining was always subtracted from specific results. In the cytometric panels shown in the Figure, the IFN-γ production by CD3 (-) cells is 1 log lower in intensity compared to the antigen-specific CD3(+) T-cell response, representing an unspecific response that may involve natural killer cells. To monitor antigen-specific T-cell responses, we collected data only from CD3(+) T cells producing higher amounts of IFN-γ. Negative control antigenic stimulation was always below the detection limit of the assay (0.02%).

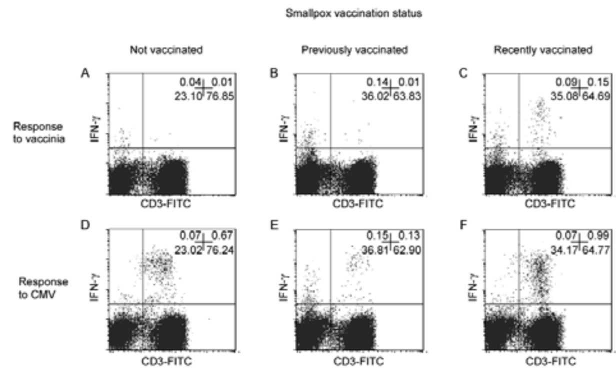

Figure. Flow cytometric analysis of T-cell responses to smallpox antigens after recent smallpox vaccination and in long-term vaccinated or not vaccinated persons. Interferon (IFN)-γ synthesis by T cells after an in vitro stimulation with vaccinia antigens was analyzed in eight healthy donors selected as recently vaccinated, long-term vaccinated, and not vaccinated persons. A representative experiment is reported in this figure. Panels A and D refer to an unvaccinated healthy donor (25-year-old white man, current neutralizing antibodies absent). A long-term vaccinated healthy person is reported in panels B and E (29-year-old white man received two doses of vaccine virus by scarification >20 years ago, current vaccinia neutralizing antibody titer of 1:8). Results from a recently vaccinated person are shown in panels C and F (31-year-old white man, single dose of Dryvax vaccine virus [Wyeth Labs, Marietta, PA] by scarification, January, 2002, current vaccinia neutralizing antibody titer of 1:32). Serum was tested for standard neutralization assay. Briefly, 0.1 mL of serial twofold dilutions of each serum was mixed with an equal volume of vaccinia virus suspension containing ~100 TCID50. After incubation, virus-antibody mixtures, medium, and virus controls were inoculated onto monolayers of Vero cells seeded in 96-well plates. Concomitant retitration of virus suspension was run in parallel. After 48-h incubation at 37°C, the cytopathic effect was observed under light microscope, and the microplates were stained with crystal violet. For T-cell assays, peripheral blood mononuclear cells cultures were stimulated with vaccinia virus (panels A–C) or cytomegalovirus antigens (panels D–F), and intracellular IFN-g synthesis was analyzed in CD3(+) T cells. Percentages in panel quadrants refer to total lymphocytes.

Cytometric panels in the Figure show the IFN-γ synthesis by CD3(+) T cells after in vitro stimulation with vaccinia virus or CMV antigens. As shown in panels D, E, and F, all donors were strongly reactive to the CMV antigens (0.87%, 0.20%, and 1.53% of CD3(+) T cells respectively; the numbers of CMV-specific CD3(+) T cells per blood milliliter were 13,132, 2,964, and 11,385, respectively). As previously described (6), most of the CMV-specific response was related to CD4(+) T cells (96%, 75%, and 59% of CMV-specific T cells, respectively). Both unvaccinated and long-term vaccinated healthy donors had undetectable responses to smallpox vaccinia antigens (Figure, panels A and B). In contrast, a recall response was detectable after a recent immunization (Figure, panel C). In this case, the percentage of T cells specific for smallpox vaccine antigens was 0.23% among CD3(+) T cell , and the number of vaccinia antigen-specific cells was 1,725 per blood mL corresponding to a frequency of 1/667. Most vaccinia-specific T cells detected by this assay were CD4(+) (vaccinia-specific CD4(+) T cells were 80% of vaccinia-specific T cells). Nevertheless, the sensitivity of this assay to detect CD8(+) T cells could be improved by using human leukocyte antigen (HLA) class I–specific peptides as previously described (4).

Conclusions

Vigorous and long-lasting protective immune responses have been associated with smallpox vaccination, and specific immunity is believed to be maintained for decades (10,11). In long-term vaccinated persons, virus-specific CD4(+) and CD8(+) T-lymphocytes are detectable only after extensive in vitro culture and restimulation to generate antigen-specific lines or clones. This limitation is due to the long, but limited, lifespan of memory T cells and to their low frequency, usually below 1/50,000 (12). Our in vitro rapid assay based on a short-time primary T-cell response was unable to show the residual memory T-cell response present in long-term vaccinated persons since the assay sensitivity is 1 log lower but could detect the higher frequencies of IFN-γ–producing antigen-specific cells appearing a few weeks after smallpox vaccine inoculation (8). Accordingly, Terajima et al. (13) demonstrated that T-cell responses to vaccinia and variola conserved epitopes peak 14 days after primary immunization with vaccinia virus. In this study, the frequency of antigen-specific T cells was measured as IFN-γ production by ELISPOT and HLA/peptide tetramer–staining methods. Because strong correlations between the data derived from ELISPOT, tetramer assays, and intracellular cytokine staining for IFN-γ were previously observed (14), vaccinia-specific T cells could be detected by flow cytometry only a few days after immunization with vaccinia virus. In addition, Pincus and Flick demonstrated the initial development of delayed hypersensitivity, an index of cell-mediated immunity, as early as 2 days after smallpox vaccination (15). During viral infection, high levels of virus-specific T cells are found in acute infection, falling below detectable limits as the viral load decreases and reappearing in chronic infections during episodes of transient viremia. Accordingly, we observed that the frequencies of HIV-specific CD8(+) T cells releasing IFN-γ were quantitatively increased a few weeks after viral rebound consequent to the interruption of antiviral therapy (5). These observations indicate that the frequency of virus-specific T cells is clinically relevant, which suggests that this method may be useful in detecting immune response by monitoring the frequency of virus-specific T cells. In recently vaccinated persons, memory cells are expanded by antigen reexposure, and their increase in frequency could be quantitatively detected by the rapid flow cytometric T-cell assay, confirming the efficacy of vaccination. Moreover, because of the high similarity between orthopoxviruses, this rapid assay using vaccinia antigens could be used to identify recently exposed persons.

Finally, an important aspect in developing a diagnostic assay is to use a rapid and easily automated system that works on virtually all persons who carry the disease. In this context, the intracellular T-cell cytokine staining by flow cytometry presents several advantages in comparison to other techniques, such as tetramer staining and ELISpot (4). In fact, flow cytometry allows for testing multiple proteins or peptides at a single time and provides at the same time a quantitative and phenotypic assessment of CD8(+) and CD4(+) responding T cells. Moreover, optimization of antigen preparation with peptide pools designed to be virus-specific, highly conserved, and independent of HLA haplotypes may allow for the development of a second generation of more sensitive flow cytometric T-cell assays, extending the possibility to perform routine analysis on cryopreserved samples (4). The technique could be easily automated through the use of analytical instruments already available in most clinical laboratories that use flow cytometry. In comparison with other analytical systems for assessing antigen-specific responses, this method is economically advantageous. The recent availability of mobile flow-cytometer units may allow use of this assay under field investigation conditions.

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Shigella dysenteriae Serotype 1, Kolkata, India

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Since July 2002, bacteriologically confirmed shigellosis cases have increased, and multidrug-resistant *Shigella dysenteriae* serotype 1 strains have reemerged in patients hospitalized with diarrhea in Kolkata, India. The isolated strains of *S. dysenteriae* 1 showed resistance to chloramphenicol (80%), ampicillin (100%), tetracycline (100%), co-trimoxazole (100%), nalidixic acid (100%), norfloxacin (100%), and ciprofloxacin (100%). Emergence of fluoroquinolone resistance in *S. dysenteriae* 1 strains complicated treatment of shigellosis patients. Six strains belonging to provisional serovars of *S. dysenteriae* were also identified for the first time in patients hospitalized with diarrhea in Kolkata, India.

Shigellosis is an important cause of bloody diarrhea in all age groups, especially in children. Of all serotypes of shigellae, *Shigella dysenteriae* type 1 attracts special attention for its epidemic-causing potential and its association with most serious dysentery cases, with a high attack rate, high case-fatality rate, and various complications (1). Antimicrobial therapy is usually recommended for treatment of shigellosis. However, antimicrobial resistance in enteric pathogens, including *Shigella* isolates, complicates the situation in developing countries, where shigellosis is endemic and indiscriminate use of antimicrobial agents is common.

During early 1984, various areas of India, including the eastern region, witnessed an extensive epidemic of bloody dysentery, predominantly caused by multidrug-resistant *S. dysenteriae* type 1, which swept through the districts of West Bengal from north to south. The strains were resistant to streptomycin, tetracycline, and chloramphenicol; highly sensitive to nalidixic acid, gentamicin, furazolidone; and moderately sensitive to ampicillin and cotrimoxazole (2). Nalidixic acid–resistant strains of *S. dysenteriae* 1 emerged in Eastern India during 1988 (3). In 1992, *S. dysenteriae* 1 was isolated from 24% of total bloody diarrhea case-patients, and the strain showed resistance to nalidixic acid (30%), furazolidone (2%), ampicillin (95%), and co-trimoxazole (88%). All strains were susceptible to fluoroquinolone derivatives, i.e., norfloxacin and ciprofloxacin (4). Therefore, furazoldione and nalidixic acid were used as first-line drugs for shigellosis during that period, with selective use of fluoroquinolones.

Changes in the worldwide epidemiology of shigellae species have been documented in the last two decades. Although bacteriologically confirmed childhood shigellosis cases varied from 4% to 6%, a change in serotypes and antimicrobial resistance in *Shigella* species was noticed in Kolkata during 1995–2000 (5). *S. flexneri* (58%) completely replaced *S. dysenteriae* (5%) and became the most prevalent serotype, followed by *S. sonnei* (28%) and *S. boydii* (9%). During 1997 to 2000, *S. dysenteriae* type 1 strain was not isolated. One strain of *S. dysenteriae,* isolated in 1999, and three strains of *S. dysenteriae,* isolated in 2000, belonged to *S. dysenteriae* type 2 (unpub. data). Isolated strains were resistant to nalidixic acid (29% with MIC_{90} <128 µg/mL), tetracycline (90%), co-trimoxazole (90%), ampicillin (67%), and chloramphenicol (46%). Again all strains were susceptible to norfloxacin (MIC_{90} <1 µg/mL) and ciprofloxacin (MIC_{90} = 0.125 µg/mL), rendering them drugs of choice for treatment of shigellosis in recent years. Routine surveillance data from National Institute of Cholera and Enteric Diseases (NICED) showed a 1% to 2% isolation rate of all *Shigella* serotypes from diarrhea patients since 1997, with the identification of a single strain of *S. dysenteriae* type 1 in 1998.

This study, performed as a continuation of routine surveillance for diarrheal diseases in two large hospitals in Kolkata, found a recent increase in patients seeking treatment for acute and severe bloody diarrhea and the reemergence of *S. dysenteriae* 1 strains with altered antibiogram.

The Study

During April–May 2002, an outbreak of bacillary dysentery was reported in the northern district of West Bengal, India, among tea garden workers. A team from National Institute of Cholera and Enteric Diseases investigated the episode, and *S. dysenteriae* 1 was found to be the sole causative agent of the outbreak (6). A similar outbreak of blood dysentery caused by *S. dysenteriae* 1 occurred during March–June 2002 in the southern part of West Bengal (7). Following these episodes, we intensified the surveillance of diarrheal diseases in two hospitals of Kolkata, India. Infectious Disease (I.D.) Hospital is the biggest hospital in Kolkata, if not in India, for admission and treatment of infectious disease cases and the Dr. B. C. Roy Memorial Children's Hospital is the only referral pediatric hospital in the state of West Bengal, which usually serves an area that includes the Kolkata metropolis and

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suburbs. In both hospitals, patients with diarrhea were kept under continuous surveillance after admission to the Diarrhoea Treatment Unit (DTU) of the hospitals and were treated with oral rehydration solution and antimicrobial agents as advised by attending clinicians.

Rectal swabs or fresh stool samples were collected from children with acute diarrhea admitted to Dr. B.C. Roy Memorial Children's Hospital, Kolkata from January 2001 through August 2002. The children were selected irrespective of type, duration of diarrhea, and history of antibiotic drug therapy.

During the first week of July 2002, an upsurge of acute bloody diarrhea cases was noticed in patients attending the I.D. Hospital, Kolkata, and increased numbers of patients with acute dysentery continued to be admitted to the hospital until September 2002. The patients reported bloody stools, abdominal pain, and tenesmus, with or without fever. Stool samples or rectal swabs were collected from all of these patients on admission from July 1, 2002, to August 31, 2002.

The samples were placed in Cary-Blair transport medium and processed within 2 hours of collection in the Microbiology Laboratory of the National Institute of Cholera and Enteric Diseases, Kolkata; the samples were tested for the entire gamut of enteropathogens by using standard techniques. *Shigella* species were confirmed with the API 20E test (Biomerieux, Marcy l'Etoile, France) and slide agglutination test with antisera specific to serotypes of *Shigella* species (Denka Seiken Co, Tokyo, Japan). Antimicrobial susceptibility testing of *Shigella* isolates was done by the disk diffusion method, and MICs were measured by using the agar dilution method.

In addition to the conventional technique, polymerase chain reaction (PCR) was performed within 4–6 hours on LB broth cultures of 77 stool samples collected from I. D. Hospital by using published primer sequences for *Ipa*H (invasion plasmid antigen H) gene to detect further *Shigella* infection, which might have been missed by conventional methods (8). PCR was also performed in anticipation of rapid diagnosis and early treatment of shigellosis cases and thus prevent the development of complications.

Table 1 shows the distribution of *Shigella* serotypes from patients admitted in two hospitals of Kolkata. An increased isolation rate of all serotypes of shigellae was observed in patients with acute diarrhea (72/790; 9.1%) and also in patients with bloody diarrhea (72/237; 30%) in the Children's Hospital since January 2002. Although *S. flexneri* continued to be the most prevalent serotype (45/72; 62.5%), followed by *S. sonnei* (18/72; 25%), the reemergence of *S. dysenteriae* serotype 1 (5/72; 7%) claimed special attention. During 2001, only one strain of *S. dysenteriae* 1 was isolated in July, but during January to August 2002, five *S. dysenteriae* 1 strains were identified: one in April and two in both July and August 2002. The other four *S. dysenteriae* strains isolated in 2001 were *S. dysenteriae* type 2 (two strains), *S. dysenteriae* type 3 (one strain), and *S. dysenteriae* type 6 (one strain).

Among 77 patients with bloody diarrhea in I. D. Hospital examined through August 2002, *Shigella* spp. were identified as the sole pathogen in 27 (35%) patients. Of 27 *Shigella* strains, 24 (88%) belonged to *S. dysenteriae* serotype 1; other strains isolated were *S. flexneri* (2 strains) and *S. sonnei* (1 strain). PCR could detect shigellae infection in 37 (48%) patients, and 12 of these patients were not culture-positive. No other pathogen could be detected in samples from any of the case-patients. The Figure shows the epidemic curve for *S. dysenteriae* 1 case-patients admitted to I.D. Hospital during the period of study.

The antimicrobial resistance profiles of the isolated *S. dysenteriae* 1 strains showed multidrug resistance to at least seven or more antimicrobial agents, e.g., chloramphenicol (80%), ampicillin (100%), tetracycline (100%), cotrimoxazole (100%), furazolidone (50%), nalidixic acid (100%), ciprofloxacin (100%), norfloxacin (100%), and amoxicillin (100%). MICs of antimicrobial agents also showed higher level of resistance acquired by these strains compared to the MICs observed during 1995–2000 (5). The $MICs_{90}$ of antimicrobial agents tested were as follows:

Table 1. Isolation frequency of *Shigella* serotypes from patients admitted to two different hospitals, Kolkata, India

Place, y, and period of sample collection	No. samples tested (n)	Strains *Shigella* isolated n (%)	*S. dysenteriae* (n)	*S. flexneri* (n)	*S. boydii* (n)	*S. sonnei* (n)
Dr. B.C. Roy Memorial Children's Hospital						
2001	1,069	80 (7.5)	5	40	9	26
Jan–April	442	26 (5.9)	1	13	3	9
May–Aug	397	39 (9.8)	4	17	5	13
Sept–Dec	230	15 (6.5)	0	10	1	4
2002	790	72 (9.1)	5	45	4	18
Jan–April	365	32 (8.7)	1	26	1	4
May–Aug	425	40 (9.4)	4	19	3	14
Infectious Diseases Hospital						
2002						
July–Aug	77	27 (35)	24	2	0	1

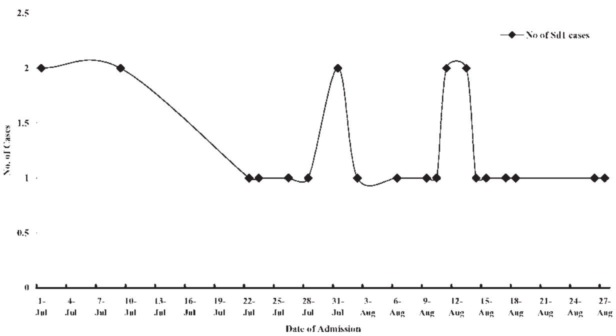

Figure. Epidemic curve for *Shigella dysenteriae* 1 case-patients admitted to Infectious Diseases, Hospital, Kolkata, India, during July and August 2002.

ampicillin (512 µg/mL), tetracycline (>256 µg/ml), chloramphenicol (<256 µg/mL), nalidixic acid (>256 µg/mL), norfloxacin (>32 µg/mL), and ciprofloxacin (>8 µg/mL). In contrast, other serotypes of shigellae were uniformly susceptible (100%) to ciprofloxacin and norfloxacin and showed partial resistance to ampicillin (60%), tetracycline (94%), cotrimoxazole (98%), and nalidixic acid (45%).

Initially, physicians advised norfloxacin and ciprofloxacin for routine treatment of shigellosis; because of poor clinical responses, subsequent patients were treated with ofloxacin to which the organism was susceptible (100%). No case fatality and no case of hemolytic uremic syndrome have been reported among the present series of patients. Median time for resolution of symptoms of the patients was 4 days from the date of admission to the hospital.

When *S. dysenteriae* 1 strains were screened for virulent gene profiles by PCR with published primer sequences (9), all *S. dysenteriae* 1 strains (100%) were found to harbor *stx*1 (Shiga toxin), *ipa*H (invasion plasmid antigen H), and *ial* (invasion-associated locus) gene and were negative for *set* (*Shigella* enterotoxin 1) gene. Sixteen (70%) of 23 isolates were positive for *sen* (*Shigella* enterotoxin 2) gene. Only one *S. dysenteriae* 1 strain showed both *Shigella* enterotoxin 1 and 2 (*set* and *sen*) genes.

Although the proportion of *Shigella* strains isolated from case-patients with acute bloody diarrhea increased (30% to 35%), the rate of *S. dysenteriae* 1 isolated from Dr. B.C. Roy Memorial Children's Hospital (5/237; 2%) was not as high as that of patients at I.D. Hospital (27/77; 35%). Because I.D. Hospital was a general hospital and provided treatment to patients of all ages, including children, concerned parents of patients who had severe dysentery caused by *S. dysenteriae* 1 might have brought them to I.D. Hospital in anticipation that their illness would be better managed.

Isolated strains of *S. dysenteriae* 1 from two recently documented outbreaks in West Bengal also showed reduced susceptibility to fluoroquinolones (6,7). In recent years, the emergence of multidrug-resistant *S. dysenteriae* 1 strains has also been reported from Southeast Asia and Africa, although fluoroquinolone resistance was not observed among the strains (10,11).

While processing the stool samples of patients admitted to the Children's Hospital for shigellae species by conventional method, we found a few strains that showed a biochemical reaction typical of *Shigella* but were nonagglutinable by commercially available antisera. These strains were positive for *ipa*H gene when tested by PCR. We designated those strains as *Shigella* untypable strains (8). The strains were sent to the Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan, for typing, and they were found to be provisional serovers of *Shigella* spp. (Table 2). Table 2 shows the year of isolation and virulent gene profiles of those strains. All strains were negative for *stx*1 gene. They were resistant to chloramphenicol, tetracycline, cotrimoxazole, furazolidone, and amoxicillin. But all strains were susceptible to nalidixic acid, norfloxacin, ciprofloxacin, gentamicin, amikacin, and cefotaxime. This drug-resistance profile contrasted with that of recently emerged *S. dysenteriae* type 1 strains. Similar strains have also been identified in other studies (12–14). To our knowledge, this is the first report of the isolation and identification of provisional serovars of *S. dysenteriae* and *S. boydii* from Kolkata, India.

Conclusions

Our study reports increased isolation of shigellae with reemergence of *S. dysenteriae* 1 in and around Kolkata, India. This increase has public health importance with respect to monitoring impending outbreaks of shigellosis and implementing appropriate strategies for containment of this deadly organism.

Emergence of multidrug-resistant *Shigella* strains is of concern to clinicians in treating shigellosis cases. Because the recently emerged *S. dysenteriae* 1 strain was resistant (100%) to ampicillin, cotrimoxazole, nalidixic acid, norfloxacin, and ciprofloxacin, which were commonly used for shigellosis cases, ofloxacin is currently recommended for treatment. A concomitant search for alternate new drugs should be continued because, although newer antimicrobial drugs can offer hope for treatment of shigellosis, emergence of resistance to the new drugs is also not far in the future. Therefore, generating an effective vaccine can offer the ultimate solution to such problems. Perhaps the more effective way of reducing the impact of the disease and the risk of contracting infection lies in improving poor living conditions, disseminating health education, and supplying safe drinking water. However, accomplishing those objectives and reaching the goal is not an easy task in developing countries. Laboratory detection capabilities also need to be strengthened at all levels to increase

Table 2. Virulence gene profiles of provisional serovars of *Shigella* spp. isolated from Kolkata, India

Serial no.	New serovars *Shigella* spp. (n)	Mo and y of isolation	Detection of virulence genes by PCR[a]				
			*Ipa*H	*ial*	*set*	*sen*	*stx*1
1.	*S. dysenteriae* 204/96	May 2000	+	+	-	+	-
2.	*S. boydii* E16553	June 2000	+	+	-	+	-
3.	*S. dysenteriae* 93-119	Oct 2000	+	+	-	+	-
4.	*S. dysenteriae* 204/96	Aug 2001	+	+	-	+	-
5.	*S. dysenteriae* E-23507	Aug 2001	+	+	-	+	-
6.	S. dysenteriae I-9809-93	Dec 2001	+	+	-	-	-
7.	*S. dysenteriae* 204/96	Mar 2002	+	+	-	+	-

[a]PCR, polymerase chain reaction; +, positive; -, negative

the baseline surveillance data for improved isolation of the pathogen. Identifying some strains with provisional serovars of *Shigella* spp. for the first time from Kolkata, India, indicates that all provisionally identified *Shigella* strains should be sent to a reference laboratory for typing and further characterization.

Studying plasmid profiles of isolated *S. dysenteriae* 1 strains and typing the strains by using various molecular tools could provide insight into the origin of these recently isolated *S. dysenteriae* 1 strains and the relationships among the strains.

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Levofloxacin Treatment Failure in *Haemophilus influenzae* Pneumonia

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We describe the first case of failure of oral levofloxacin treatment of community-acquired pneumonia caused by *Haemophilus influenzae*. The strain showed cross-resistance to fluoroquinolones and carried four mutations in quinolone resistance–determining regions of DNA gyrase and topoisomerase IV genes.

Fluoroquinolones active against *Streptococcus pneumoniae* and *Haemophilus influenzae,* such as levofloxacin, moxifloxacin, and gatifloxacin, are currently recommended for the treatment of patients with community-acquired pneumonia whose infection may be due to strains resistant to β-lactam and macrolide antimicrobial drugs (1–3). In Spain, both pathogens often display multiple resistance (4,5). The failure of oral levofloxacin treatment in four patients with pneumococcal pneumonia has recently been reported (6).

We describe failure of treatment of *H. influenzae* pneumonia in a patient who died after several days of systemic treatment with levofloxacin. The *H. influenzae* strain, isolated from the blood and the respiratory tract of the patient, was resistant to levofloxacin, ciprofloxacin, moxifloxacin, and gatifloxacin and carried several mutations in the quinolone resistance–determining regions (QRDR) of DNA gyrase and topoisomerase IV genes.

Case Report

On February 15, 2002, a 71-year-old woman was admitted to Hospital de l'Esperit Sant, Santa Coloma de Gramenet, Barcelona, with cough, low-grade fever, and reported fatigue. A radiograph of her chest showed infiltrates covering the right upper, middle, and lower lobes and the left basal lobe. A systemic, antimicrobial treatment of levofloxacin (500 mg/day) and teicoplanin (400 mg/day) was administered for severe community-acquired pneumonia. (The patient was allergic to penicillin.) Three months earlier, she had received erythromycin. In a second visit, she was treated with inhaled salbutamol and glucocorticoids. In a third visit, 2 months before she became ill, she received oral moxifloxacin for 5 days.

During her hospital stay, the patient's clinical condition worsened. Ventilation and perfusion scintiphotographs with Tc99m, an echocardiograph, and a high resolution thoracic scan were performed in an effort to identify another illness or a possible nondrained focus; results were negative. After 7 hospital days of systemic levofloxacin therapy, her clinical condition had not improved. Two blood cultures taken after an episode of fever with a temperature of 38.5°C showed that *H. influenzae* was resistant to fluoroquinolones (strain 32602). Treatment was switched to chloramphenicol (1 g/6 h), to which the strain was susceptible. However, on the same day as the treatment change, the patient was intubated during a severe episode of respiratory impairment and transferred to the intensive care unit of Hospital de l'Hospitalet, L'Hospitalet de Llobregat, Barcelona. Fluoroquinolone resistance was confirmed with blood cultures (strain 35102) and a bronchial aspirate (strain 35202). Antimicrobial treatment was then switched to aztreonam (1 g/8 h). The patient died 48 hours later.

The Study

Species identification, biotyping, and serotyping followed recommended protocols (7), including capsule genotyping of six capsular types (types a–f). Susceptibility testing was performed by microdilution methods, following the guidelines of the National Committee for Clinical Laboratory Standards (8). β-lactamase activity was determined by the chromogenic cephalosporin test with nitrocephin as substrate. Quality control strains used were *H. influenzae* ATCC 49247, *H. influenzae* ATCC 51907, and *Escherichia coli* ATCC 25922. Determination of an eventual active efflux mechanism of fluoroquinolone resistance was carried out by MIC determination in the presence and absence of carbonyl cyanide 3-chlorophenyl (CCCP) and reserpine in all three clinical strains (9).

The three test strains and four additional *H. influenzae* biotype II isolates (obtained previously from blood and respiratory specimens from the geographic area where the patient lived) were examined by pulsed-field gel electrophoresis (PFGE) after digestion of bacterial DNA with *Sma*I (MBI Fermentas, Vilnius, Lithuania) and separation of the fragments with the CHEF Mapper apparatus (BioRad Laboratories, Hercules, CA) and were visually compared (10).

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Amplification and sequence analysis of the QRDRs of *gyrA, gyrB, parC,* and *parE* was performed in the three clinical test isolates and in the reference strain *H. influenzae* ATCC 51907 (11). These specific primers were used: *gyrA*-f (5′-CCGCCGCGTACTATTCTCAAT-3′), *gyrA*-r (5′-GTTGCCATCCCCACCGCAATACCA-3′), *gyrB*-f (5′-CCTGCTCTTTCTGAAACTTTAC-3′), *gyrB*-r (5′-CCATCTAACGCAAGGGTTAATC-3′), *parC*-f (5′-tct-gaacttggcttaattgcc-3′), *parC*-r (5′-GCCACGACCTTGCT-CATAAAT-3′), *parE*-f (5′-TCGTTAGTGGCCCTGCAT-TAC-3′), and *parE*-r (5′-GAACAGGGCACAGAG-TAGGGT-3′). Sequencing was done on both DNA strands with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The products were resolved and analyzed with an ABI PRISM 377 DNA sequencer (Applied Biosystems). Nucleotide sequences were analyzed by using sequence analysis software (DNAstar, Inc., Madison, WI).

The three clinical isolates were identified as noncapsulated *H. influenzae* biotype II. The PFGE yielded the same pattern for each, unlike the marked diversity obtained with the four other clinical isolates from the same geographic area (Figure), and the same antimicrobial drug susceptibility testing results (Table). The results of DNA sequencing of the QRDRs of the *gyrA*, *parC, parE,* and *gyrB* genes are shown in the Table. All three strains presented identical amino acid substitutions. They did not produce β-lactamase and were fully susceptible to several β-lactam antimicrobial drugs and chloramphenicol. MICs of additional antimicrobial drugs were penicillin, 0.25 µg/mL; ampicillin, 0.12 µg/mL; amoxicillin-clavulanic acid, 0.12/0.06 µg/mL; cefuroxime, 0.12 µg/mL; cefotaxime, 0.03 µg/mL; aztreonam, 0.03 µg/mL; chloramphenicol, 0.12 µg/mL; erythromycin, 16 µg/mL; clarithromycin, 16 µg/mL; and azithromycin, 8.0 µg/mL. Quinolones, other than those depicted in the Table, had the following activity levels: ofloxacin, 32.0 µg/mL; grepafloxacin, 32 µg/mL; clinafloxacin, 2.0 µg/mL. No changes in the MICs of fluoroquinolones were observed either in the presence or absence of CCCP or reserpine, suggesting that active efflux did not contribute to fluoroquinolone resistance in any of the isolates.

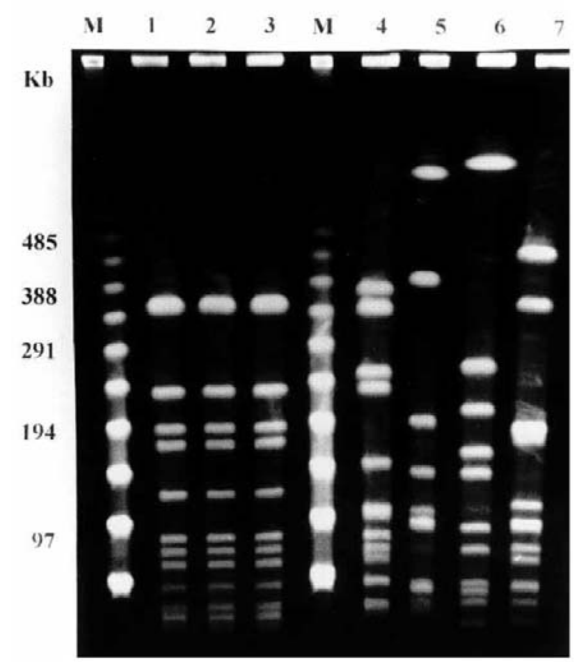

Figure. Pulsed-field gel electrophoresis patterns of the three clinical isolates of levofloxacin-resistant *Haemophilus influenzae* from the case report (lanes 1 to 3) and four control cases of levofloxacin-susceptible *H. influenzae* biotype II strains isolated from the same geographic area (lanes 4 to 7). Lane 1: strain 32602 (blood); lane 2: strain 35102 (blood); lane 3: strain 35202 (bronchial aspirate); lane 4: pleural fluid isolate; lane 5: blood isolate; lane 6: pleural fluid isolate. M, molecular markers.

Conclusions

This study is the first description of a clinical case of a fluoroquinolone-resistant *H. influenzae* pneumonia and bacteremia, resulting in therapeutic failure and death after systemic levofloxacin treatment. The causative strain was present in cultures from the patient's blood and bronchial aspirate. This finding is consistent with the interpretation of the initial chest x-rays, which indicated a clinical case of severe community-acquired pneumonia. Similar cases of community-acquired pneumococcal pneumonia treated with levofloxacin have recently been reported (6).

We have previously described the emergence of fluoroquinolone-resistant *H. influenzae* in the upper respiratory tract of patients with chronic respiratory infections treated with oral fluoroquinolones (7,12). However, in this patient the only documented exposure to a fluoroquinolone was a

Table. Fluoroquinolone susceptibility and amino acid changes in GyrA, ParC, GyrB, and ParE QRDR fragments of three *Haemophilus influenzae* strains compared with a fully fluoroquinolone-susceptible strain[a]

	MIC (µg/mL)				Amino acid at specified positions		
Strain no.	CIP	LEV	MOXI	GATI	GyrA	ParC	ParE
ATCC 51907	0.007	0.03	0.01	0.003	84 Ser 88 Asp	84 Ser	420 Asp
32602, 35102, 35202	16	16	8	16	Leu Asn	Ile	Asn

[a]QRDR, quinolone resistance-determining regions; CIP, ciprofloxacin; LEV, levofloxacin; MOXI, moxifloxacin; GATI, gatifloxacin. Microdilution used for susceptibility testing (8).

5-day treatment with moxifloxacin 2 months before the pneumonia appeared. This case suggests that exposure to a fluoroquinolone may contraindicate the use of another fluoroquinolone; however, this proposal may not be practical, especially in a patient who is allergic to penicillin.

We have already established that amino acid substitutions in the QRDRs of DNA gyrase and topoisomerase IV accounted for the mechanism of fluoroquinolone resistance in *H. influenzae* (12).The positions and the amino acid substitutions found in GyrA and ParC in this study were also present in the fluoroquinolone-resistant strains previously studied by our group (12). We have also described the Asp420Asn substitution (ParE) in another strain that exhibited high resistance levels to ciprofloxacin and other fluoroquinolones (13). However, the pattern of QRDR mutations found in the clinical strains described in this study is novel.

Use of the "mutant prevention concentration" has been proposed to discourage the selection of fluoroquinolone-resistant mutants, since resistant mutants are selected exclusively within a concentration range known as the "mutant selection window" (14). This "window" may be more easily attained with therapeutic regimes based on oral and once-daily administered doses, as is the case for moxifloxacin and levofloxacin. An important difference between *S. pneumoniae* and *H. influenzae* is that the MIC_{90}'s for levofloxacin are 1.0-2.0 µg/mL and 0.03 µg/mL, respectively (3), a 30-fold difference. This suggests that the events necessary to reach a similar level of resistance in *H. influenzae* are more complex than they are in *S. pneumoniae*.

The peak level of levofloxacin, 4 hours after a 500-mg dose, is approximately 4.0 µg/mL (serum) and 11.0 µg/mL (epithelial fluid) (3,15), which is clearly below the MIC of 16.0 µg/mL shown by these resistant strains. Where these clinical *H. influenzae* strains originated is unknown. Following the patient's oral moxifloxacin treatment 2 months before hospital admission, the *H. influenzae* may have become resistant to fluoroquinolones through a typical, step-by-step mutation process in the primary targets, DNA gyrase and topoisomerase IV, by means of four mutations in *gyrA*, *parC*, and *parE*, genes. The initial treatment also may have resulted in the mutations in *gyrA* only, with additional mutations occurring during the subsequent treatment with levofloxacin.

The *H. influenzae* strain acquired several mutations in the QRDRs and was cross-resistant to all members of a group of assayed fluoroquinolones. We believe that this case may represent an emerging clinical and microbiologic challenge, which may threaten the management of pneumonia and other infections. We recommend that blood cultures taken from patients with community-acquired pneumonia be tested for fluoroquinolone resistance, particularly when the patient has already been given these antimicrobial drugs.

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The DNA sequences obtained in this study have been submitted to GenBank under accession nos. AJ508043, AJ508044, AJ508045, and AJ508046.

Dr. Bastida is a clinical microbiologist in charge of a diagnostic microbiology laboratory in a 165-bed hospital. Her research interests are focused in clinical microbiology.

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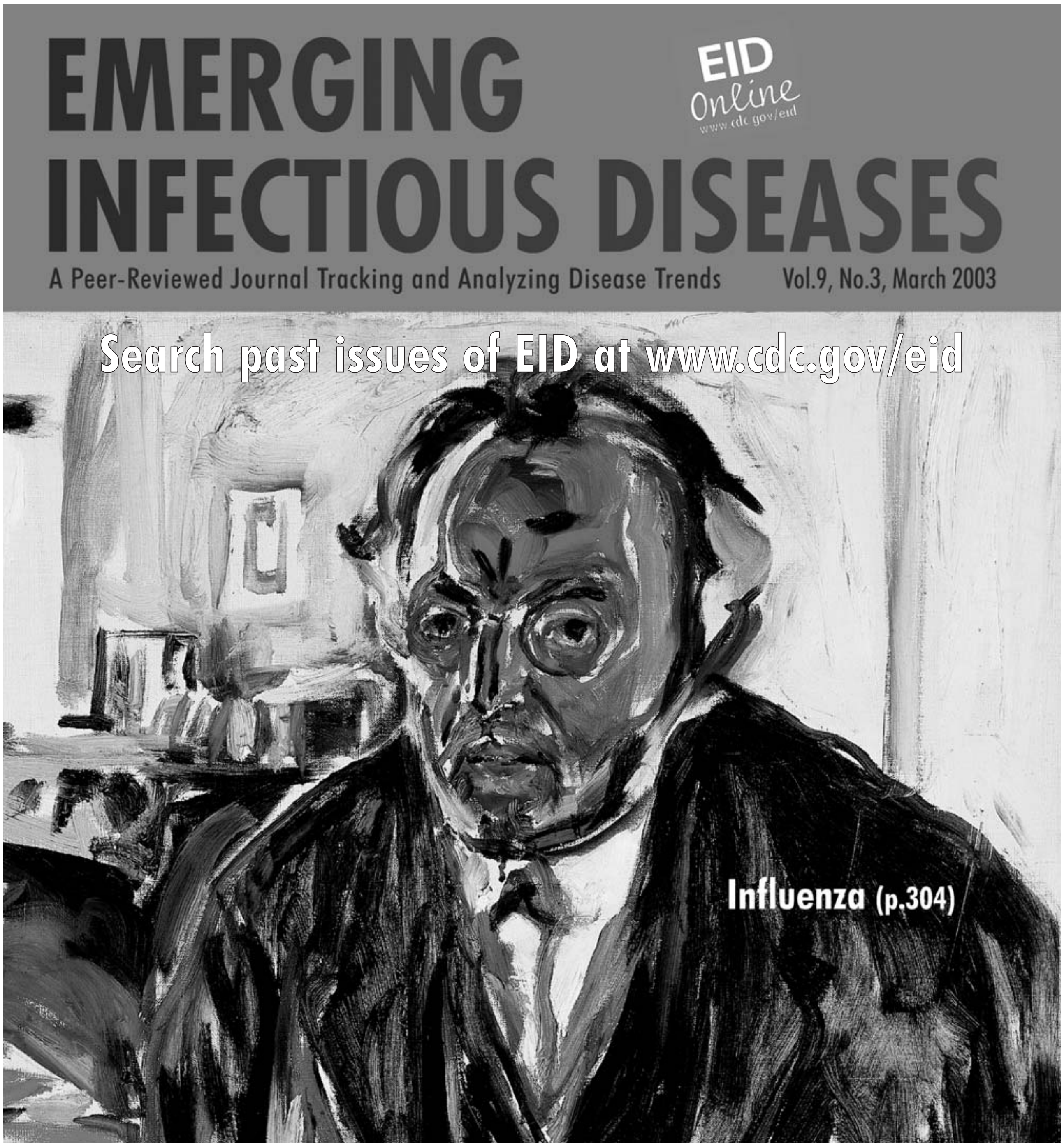

Fluoroquinolone Susceptibility of *Campylobacter* Strains, Senegal

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To assess fluoroquinolone susceptibility of *Campylobacter* strains in Senegal, skin samples were collected from 250 chicken carcasses from January 2001 to October 2002. Among 205 isolated *Campylobacter* strains, 59% and 41% were identified as *Campylobacter jejuni* and *C. coli*, respectively; the overall ciprofloxacin-resistance rate was 34%.

Campylobacter species are one of the most common causes of human bacterial diarrhea in industrialized and developing countries (1–3). *Campylobacter jejuni* and *C. coli* are the two main species involved in human infections (1,2). Because of genotyping and serotyping analysis, several studies have confirmed that poultry can be a source of *Campylobacter* infection in humans (1,4). The contamination may be due to direct ingestion of undercooked food or cross-contamination of raw poultry to other foods by nonhygienic handling (e.g., unwashed hands, dirty utensils) (1).

No antimicrobial drug therapy is usually required for *Campylobacter* infections, since they are of short duration, clinically mild, and self-limiting. However, antimicrobial drug treatment is indicated for severe infections or persons at risk such as children or immunocompromised patients (1,2), especially in Africa where AIDS has reached epidemic proportions.

Fluoroquinolones are effective synthetic antimicrobial drugs used for treatment of a wide variety of bacterial infections, including campylobacteriosis (5). Because of heavy use of fluoroquinolones in veterinary medicine during the 1990s, numerous fluoroquinolone-resistant *Campylobacter* strains are emerging both in animals and in humans (1). We report the first evidence of ciprofloxacin-resistant *Campylobacter* strains isolated from chickens in Senegal, West Africa.

The Study

From January 2001 to October 2002, a total of 250 chicken carcasses were collected in Dakar, the capital of Senegal, and its suburbs. Chicken carcasses originated from broiler chickens from semi-industrialized areas. These chickens had been raised domestically; no imported animal was included in the study. Chicken carcasses were selected from a random sampling of 80 retail shops and slaughterhouses. In Senegal, the slaughtering process is manual, rudimentary, and often performed by a single person outside in poor hygienic conditions. Each carcass was examined for *Campylobacter*.

Standard methods for isolating *Campylobacter* from the skin were used, and species identification was performed by polymerase chain reaction (4). One colony per positive culture was randomly selected. The strains were maintained as stock culture in glycerol at –80°C and tested further by the E test method for susceptibility to ciprofloxacin (6). As previously reported for *Campylobacter*, the breakpoint used for resistance was $\geq$4 µg/mL for ciprofloxacin (7). The chi-square test was used for statistical analysis (EpiInfo, version 6, Centers for Disease Control and Prevention, Atlanta, GA). A p value of <0.05 was considered statistically significant.

Campylobacter were isolated from 82% of the samples: 120 *C. jejuni* and 85 *C. coli* were identified. MIC performed by E test showed that 34% of *Campylobacter* strains were resistant to ciprofloxacin without a significant difference between the two species (Table): 71% and 79% of *C. coli*– and *C. jejuni*–resistant strains, respectively, displayed a ciprofloxacin MIC $\geq$32 µg/mL. These rates were also statistically similar and showed a high level of resistance to ciprofloxacin in strains isolated in Dakar (Table).

Our data showed a significant isolation rate of *Campylobacter* strains (82%). In every retail shop and slaughterhouse, at least one chicken in the three or four randomly selected harbored a *Campylobacter* strain. As previously reported (8), *C. jejuni* was more frequently isolated than *C. coli* (59% vs. 41%). During the microbiologic analysis, one colony per culture was selected and iden-

Table. Distribution of ciprofloxacin MICs for 85 *Campylobacter coli* and 120 *C. jejuni* isolates from broiler carcasses in Senegal

Ciprofloxacin MIC (ì g/mL)	*C. coli*: No. of strains	*C. jejuni*: No. of strains
0.032–0.063	26	34
0.064–0.124	23	27
0.125–0.249	5	18
0.250–0.4	0	1
0.5–0.99	0	1
1.00–1.99	0	0
2.00–3.99	0	0
4.00–7.99	4	5
8.00–15.99	4	2
16.00–32.00	1	1
>32.00	22	31
Total	85	120

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tified. Since different strains and even species of *Campylobacter* can frequently be isolated from poultry products (7), the number of species isolated in our survey might be underestimated.

Conclusions

High levels of *Campylobacter* isolation from chicken carcasses and retail chickens have been previously reported in industrialized countries: 46% in Germany (9), 46% in Japan (10), and 73% to 100% in the United States (11). Although little information is available from developing countries, our results are consistent with those from Kenya, where thermophilic *Campylobacter* organisms were isolated from 77% of chicken samples (12). These results showed that the *Campylobacter* prevalence in chicken carcasses in some African countries may be similar to those observed in industrialized countries.

A high frequency of ciprofloxacin-resistant *Campylobacter* strains was observed in our survey. The prevalence of resistant strains was similar for *Campylobacter* isolated from chickens in Lebanon (13), the United States (11), and Japan (14). The prevalence was lower than the rate of resistant strains from Belgium (7) or Spain (2), where 100% of *C. coli* and 98.7% of *C. jejuni,* respectively, were fluoroquinolone resistant. In Taiwan, Thailand, and Spain, fluoroquinolone-resistance rates, ranging from 56% to 84%, were similar in poultry meat and human isolates (1,2). By contrast, in some countries such as Chile, no ciprofloxacin-resistant *Campylobacter* strains have been described (6).

Since 1991, when Endzt and colleagues (15) identified the first quinolone-resistant *Campylobacter* strains in *C. jejuni* and *C. coli* in the Netherlands, *Campylobacter* resistance to fluoroquinolones has increased throughout the world (2,6,8). This important development in humans and other animals is related to the introduction of fluoroquinolones in veterinary medicine (15). In Senegal, fluoroquinolones (enrofloxacin, norfloxacin) were introduced in veterinary medicine in 1996 in poultry production to treat respiratory and intestinal diseases. Because of the absence of any antibiotic resistance studies in Senegal, correlating the currently observed ciprofloxacin resistance with the introduction of ciprofloxacin into the country is difficult. However, our results show, as in industrialized countries, that fluoroquinolone resistance may become a public health problem for developing countries. Further studies are needed to evaluate how commonly fluoroquinolone resistance occurs among human isolates of *Campylobacter* in Senegal, and to what extent poultry contributes.

Because of their wide treatment spectrum, which includes bacterial infections such as salmonellosis and shigellosis, fluoroquinolones have progressively become the empiric treatment for human gastroenteritis in Senegal. In the region of Dakar, fluoroquinolones are used in all facets of poultry production, including for chickens used for broiling, laying, and breeding. Fluoroquinolones also tend to be the most prescribed drug for treatment of respiratory and intestinal diseases among poultry (approximately 25% of the antimicrobial drugs delivered in veterinary use; C. Biagui, unpub. data). To decrease ciprofloxacin-resistant *Campylobacter* and preserve the effectiveness of fluoroquinolones, their use in veterinary medicine should be more strictly controlled. Our finding of fluoroquinolone-resistant *Campylobacter* in chickens suggests the epidemiology of fluoroquinolone-resistant *Campylobacter* in Senegal is the same as in other countries.

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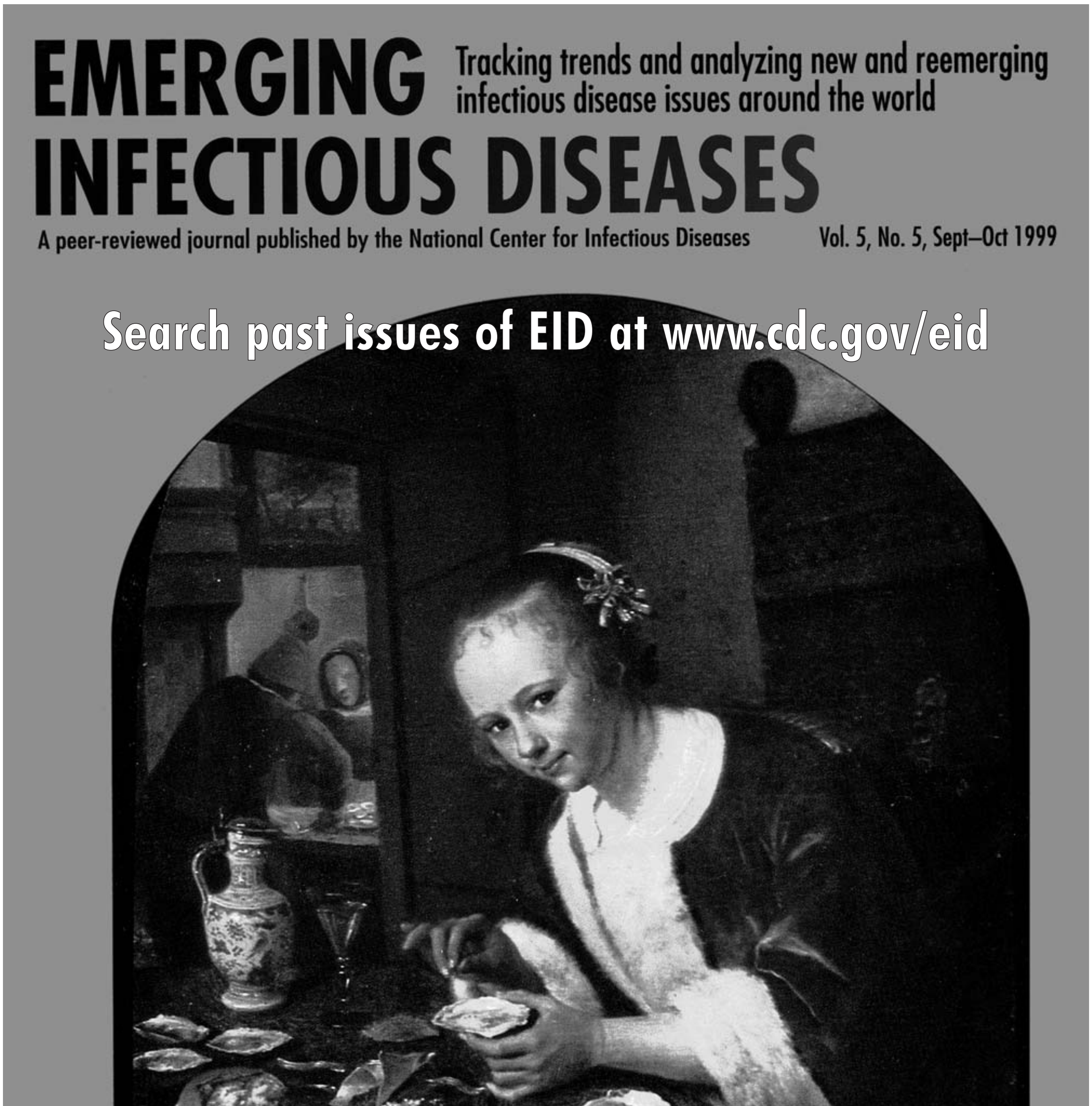

Fluoroquinolone Resistance in Campylobacter Absent from Isolates, Australia

Leanne Unicomb,* John Ferguson,† Thomas V Riley,‡ and Peter Collignon§

Fluoroquinolone resistance was detected in 12 of 370 Australian human *Campylobacter* isolates; 10 of these were travel-associated, and for 2 isolates travel status was unknown. No resistance was found in isolates known to be locally acquired. In Australia, fluoroquinolones have not been licensed for use in food production animals, a policy that may have relevance for countries with fluoroquinolone-resistant *Campylobacter*.

In Australia, *Campylobacter* is the most commonly reported bacterial foodborne pathogen with an annual incidence of 125/100,000 population (1). Fluoroquinolone resistance in this pathogen is recognized as an emerging public health problem related to the use of these antimicrobial agents in food production animals. Data from many regions (United States, Europe, and Thailand) that have licensed fluoroquinolones for therapeutic use in animals have shown that such use results in the emergence of fluoroquinolone resistance in *Campylobacter jejuni* and *C. coli* isolates obtained from both humans and animals (2–4). Increasing resistance in campylobacter may lead to infections that are unresponsive to antimicrobial drug treatment and more severe disease. Smith (2) demonstrated that resistant *C. jejuni* caused more prolonged diarrhea in patients than susceptible strains.

Fluoroquinolone resistance may emerge during treatment in humans (3); however, Smith (2) and others (5) demonstrated that most detected resistant isolates come from patients who have not been exposed to fluoroquinolones. Furthermore, as human-to-human transmission of campylobacter is rare, patients infected with resistant campylobacter are not an important source of resistance for other humans (4).

In Australia, fluoroquinolones have never been licensed for use in food production animals. A small amount is used in companion animals: imports of enrofloxacin began in 1995, and 49 kg was used in the financial year 1996–97 (6). In contrast, the average use of quinolones in humans was 3,200 kg per year from 1992 to 1997 (6).

Australian data on fluoroquinolone resistance in human *Campylobacter* isolates are limited. As part of a case-control study of risk factors for *Campylobacter* infection conducted in New South Wales from 1999 to 2001, patients infected with this pathogen were recruited, and information on various exposures was obtained by telephone interview. Patients were asked about local and international travel in the 4 weeks before onset of diarrhea. Isolates from patients were stored and subsequently tested for resistance to 10 antimicrobial agents by using the National Committee for Clinical Laboratory Standards method for *Helicobacter* species (7). The Table shows proportions of fluoroquinolone-resistant isolates from this case-control study. In addition, results of two laboratory-based surveys of antimicrobial resistance, one conducted on isolates from Western Australia and one conducted on isolates from the Australian Capital Territory, are included. In these last two studies, information on overseas travel was sought retrospectively. Fluoroquinolone-resistant human Campylobacter isolates were rarely detected in Australia. All ciprofloxacin-resistant isolates detected in the three regions were from patients who appeared to have acquired their infection outside the country (Table). Two locally acquired isolates in the New South Wales study were resistant to nalidixic acid only (i.e., they were sensitive to fluoroquinolones).

As Campylobacter infection is zoonotic, the absence of human, locally acquired infections attributable to fluoroquinolone-resistant organisms most likely reflects 1) the lack of use of fluoroquinolones in Australian poultry (the most common source for *C. jejuni*) and other potential meat sources and 2) the presence of little or no viable *Campylobacter* organisms on imported chicken, which has been a source of resistant campylobacter infections in the United Kingdom (5). Only cooked chicken products can be imported into Australia.

Fluoroquinolones are critical therapeutic agents for many serious bacterial infections because, in many cases, they may be the only active oral agents available. Resistance following fluoroquinolone use can develop in many gram-negative bacteria (campylobacter, salmonellae, and *Escherichia coli*) carried by animals. These bacteria can be present in food. If they subsequently cause infections in humans (or transfer their resistance genes to other bacteria), no effective antimicrobial agents may be available for treatment when serious disease occurs. Thus, their use in animals should be avoided. Australia has never licensed the use of fluoroquinolone agents in livestock. In contrast with other nations that have licensed their use,

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Table. Fluoroquinolone resistance data for Australian *Campylobacter* isolates

Study location	Isolate source and collection period	Total no. tested	Proportion[a] of fluoroquinolone-resistant isolates (%)		
			Locally acquired	Overseas acquired	Unknown acquisition status
New South Wales	Human feces 1999–2001	180[b]	0/144 (0)	3[c]/7 (43)	2[c]/29 (6.9)
Western Australia	Human feces 1999–2000	50[b]		4[c]	0/46 (0)
Australian Capital Territory	Human feces/blood 2001–2002	140[d]		3	0/137 (0)

[a]No. of resistant isolates by acquisition status/total no. isolates tested in acquisition status category.
[b]Testing by agar dilution, Mueller-Hinton agar with 5% lysed sheep blood (7).
[c]Resistant to ciprofloxacin (MIC $\geq$4 mg/L).
[d]Testing by disc-susceptibility method (8).

fluoroquinolone resistance in *Campylobacter* isolates and subsequent infections in humans acquired from meats eaten within the country have not emerged in Australia. The Australian experience has implications for the continued licensing of these agents in other countries for food production animals.

Antimicrobial testing of isolates from the New South Wales case-control study was funded by OzFoodNet, enhanced surveillance program of the Department of Health and Ageing, Australia.

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Cutaneous Melioidosis and Necrotizing Fasciitis Caused by *Burkholderia pseudomallei*

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In areas where melioidosis is endemic, stress on the healthcare system is substantial. Because clinical manifestations are protean, the illness is difficult to diagnose, and cutaneous *Burkholderia pseudomallei* infections can progress to necrotizing fasciitis. While it is an uncommon complication of cutaneous melioidosis, necrotizing fasciitis is potentially fatal and requires aggressive management, including early diagnosis, appropriate antibiotics selection, and operative débridement.

Melioidosis is a disease caused by a gram-negative bacterium, *Burkholderia pseudomallei*. Endemic to Southeast Asia, Taiwan, China, Central and South America, and northern Australia, sporadic infections occur throughout the world, usually in travelers to the disease-endemic areas (1–3). Humans are usually infected by soil contamination of puncture wounds. The respiratory tract is the most common site of infection (3). Cutaneous melioidosis with necrotizing fasciitis is rare: we found no reports of cutaneous melioidosis with necrotizing fasciitis and only one previous report of melioidosis with necrotizing fasciitis, which complicated postoperative drainage of a parotid abscess (4).

The Study

A 61-year-old Chinese man with a history of psoriasis and alcoholic liver cirrhosis sought treatment for left ankle swelling, erythema, and tenderness. He worked as a dishwasher in a restaurant and could not recall any antecedent trauma to the affected limb. He was febrile on admission and was empirically treated with intravenous penicillin and cloxacillin for cellulitis. Despite antimicrobial drug therapy, his lower limb infection progressed with formation of cutaneous blisters. A decision for wound débridement was made. During surgery, grayish necrotic fascia, extensive subcutaneous tissue necrosis, loss of resistance of the normally adherent superficial fascia to blunt dissection, and foul-smelling "dishwater" pus were noted. A diagnosis of necrotizing fasciitis was made.

The tissue specimen was spread onto blood agar plate (trypticase soy agar supplemented with 5% sheep blood, BBL [Becton, Dickinson and Company, Franklin Lakes, NJ]) and Ashdown's media, a selective media for *B. pseudomallei*. Characteristic colonial morphologic findings of *B. pseudomallei* were noted on blood agar and Ashdown's media. The organism's identity was confirmed with biochemical tests (positive oxidase reaction, simmons citrate) and microscopic morphologic findings. (The automated blood culture system used was the BACTEC Fluorescent Series blood culture system [Becton, Dickinson and Company].) Tissue (taken during surgery) and blood cultures grew *B. pseudomallei* susceptible to ceftazidime, amoxicillin/clavulanic acid, imipenem, chloramphenicol, and cotrimoxazole but resistant to ampicillin, amikacin, and gentamicin. Results of melioidosis serologic testing were also positive. The patient's chest roentgenogram was normal. A computed tomography scan of his abdomen and pelvis showed splenic abscesses, but no prostatic abscess was noted. A digital rectal examination demonstrated an enlarged prostate that was nontender. The patient underwent two further operations for wound débridement and split thickness skin grafting. He was treated with ceftazidime and doxycycline; when his fever persisted, his treatment was converted to intravenous imipenem. He responded well and was discharged after he completed a 4-week course of imipenem. On discharge, no oral antimicrobial drugs were dispensed.

One month later, the man was readmitted with fever associated with scrotal swelling and pain. On examination, a firm, tender right prostatic nodule was noted. A transrectal ultrasound showed abscesses in both prostatic lobes. The aspirate from the prostatic abscesses and blood cultures grew *B. pseudomallei* with antimicrobial drug susceptibilities identical to the organism cultured from his initial admission. A 6-week course of intravenous ceftazidime was given. He also underwent transurethral resection of the prostate; the histologic results indicated benign prostatic hyperplasia with acute on chronic inflammation. On discharge, he was prescribed oral amoxicillin-clavulanate (for 4 weeks) and doxycycline (for 1 year).

Seventeen months after cessation of oral antibiotics, he reappeared with a left temporal scalp abscess and fever. No organism was isolated from the scalp pus culture, but the blood culture again grew *B. pseudomallei* with identical antimicrobial drug susceptibilities. He was given 4 weeks of ceftazidime followed by amoxicillin-clavulanate (for 4 weeks) and doxycycline (long-term). After 8 months on oral doxycycline, the patient was symptom-free.

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Conclusions

This patient history is representative of the protean clinical features of melioidosis. The disease has been called the great mimicker, with a wide range of clinical syndromes including pneumonia, visceral abscesses, soft tissue infections, septic arthritis, and septicemia (1,3,5). Lim et al. previously reported a case of melioidosis with a parotid abscess that was drained and subsequently progressed to necrotizing fasciitis (4). Although not previously reported, necrotizing fasciitis can be the initial sign of melioidosis. While cutaneous infections by *B. pseudomallei* are usually indolent soft tissue infections manifesting as cellulitis or abscesses (1–3,6), these soft tissue infections can progress to necrotizing fasciitis in melioidosis.

Melioidosis can remain latent for prolonged periods and reactivate with severe sepsis years after the initial infection (1,7). Melioidosis is characterized by relapses or recrudescence, especially in immunocompromised patients (1,7,8). For this patient, the first relapse occurred 1 month after the first hospital discharge. Another relapse occurred 17 months after cessation of oral maintenance antimicrobial drug therapy.

For patients with a known history of melioidosis, antimicrobial agents for *B. pseudomallei* should be used. *B. pseudomallei* are frequently intrinsically resistant to many antimicrobial agents, including aminoglycosides and first- or second-generation cephalosporins. Current recommended therapy of severe melioidosis includes intravenous ceftazidime or imipenem for 10 days to 4 weeks, followed by maintenance oral antimicrobial agents (amoxicillin-clavulanate or a combination of trimethoprim-sulfamethoxazole and doxycycline) for 10 to 18 weeks (9).

In the context of soft tissue infections, patients with a history of melioidosis developing cutaneous septic foci should be treated promptly and aggressively with antibiotics against *B. pseudomallei* in addition to the common organisms responsible for these infections such as *Staphylococci aureus* and streptococcal species. In this setting, an intravenous regime consisting of crystalline penicillin and cloxacillin may not be the appropriate initial antimicrobial drug regime (3). Awareness that soft tissue infection can progress to necrotizing fasciitis, a life- and limb-threatening condition, is important, and a high index of suspicion should be maintained. When necrotizing fasciitis is suspected, magnetic resonance imaging of the affected area should be performed to ensure early diagnosis, and aggressive débridement of all nonviable tissue should follow for an improved outcome (10).

Melioidosis is a major problem and a rampant disease in rural parts of Central and South America, North Australia, and Southeast Asia. In northeast Thailand, it is responsible for 20% to 40% of deaths from community-acquired septicemia (11). Its true incidence is probably underestimated due to under detection and limited availability of culture facilities (12). Awareness of the spectrum of soft tissue infections caused by *B. pseudomallei,* including the distinct possibility of necrotizing fasciitis at the extreme of the spectrum, is important. The successful management of necrotizing fasciitis requires appropriate antimicrobial drug selection and aggressive operative débridement.

Acknowledgment

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Polymerase Chain Reaction Assay and Bacterial Meningitis Surveillance in Remote Areas, Niger

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Ali Djibo,‡ Kiari Kaka Kairo,‡
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To compensate for the lack of laboratories in remote areas, the national reference laboratory for meningitis in Niger used polymerase chain reaction (PCR) to enhance the surveillance of meningitis caused by *Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae*. PCR effectively documented the wide geographic spread of *N. meningitidis* serogroup W135.

Niger is a sub-Saharan country located in the center of the African "meningitis belt," which includes all or parts of 18 sub-Saharan countries (1). The population of approximately 11 million is mainly concentrated in the southern part of the territory; 84% of the population lives in rural areas. Every year, during the dry, warm, and windy season, between January and May, localized epidemics of meningococcal meningitis occur in several districts, whereas major epidemics, affecting the entire territory, break out at regular intervals of every few years (2,3). Previously, all important epidemics have been caused by *Neisseria meningitidis* serogroup A, and occasionally, small outbreaks have been caused by serogroup X isolates (4). However, sporadic cases of meningitis caused by serogroup *N. meningitidis* W135 have been laboratory-confirmed in Niger since the beginning of the 1980s (5). Strains of serogroup W135 were found as often as strains of serogroup A at the end of the epidemic of 2001 (6). In 2002 and again in 2003, Burkina Faso experienced the first large epidemics caused by *N. meningitidis* serogroup W135 in Africa (7,8). This event is a cause of concern for public health authorities of neighboring countries, including Niger, and for the World Health Organization because current affordable meningococcal vaccines available in large amounts target only *N. meningitidis* serogroups A and C and do not protect against serogroup W135. On the other hand, vaccines including the *N. meningitidis* serogroup W135 valency are not only expensive but also rare, and this situation will likely continue for the next 3 to 4 years. Enhanced microbiologic surveillance is needed to quickly identify the serogroup of *N. meningitidis* involved so that the appropriate vaccine for emergency mass vaccination can be selected (9).

In Niger, few laboratories are able to perform etiologic diagnosis of bacterial meningitis, although the country is large (1,267,000 km^2) and the population is scattered. Until 2002, microbiologic surveillance existed but was inadequate because it focused almost entirely on the capital city. The Centre de Recherches Médicales et Sanitaires (CERMES) in Niamey became, in 2002, a national biomedical research center under the authority of the Ministry of Health (MOH) and the national reference center for meningitis in Niger. The polymerase chain reaction (PCR) method for the diagnosis of acute bacterial meningitis was transferred from the Institut Pasteur (Paris, France) for research purposes in October 2002. Therefore, CERMES decided to include the PCR assay in the national framework of the routine surveillance system of the MOH in November 2002 to be ready when the next meningitis season began in January. The MOH has asked physicians and nurses to systematically keep frozen (or at least refrigerated in small health facilities) every sample of cerebrospinal fluid (CSF) collected from patients with suspected cases of acute meningitis. Most often, in remote healthcare centers and in the absence of laboratories, the clarity of the CSF was assessed by macroscopic examination only, so the entire volume of CSF that was previously discarded was kept for PCR analysis. In the few district hospitals that have appropriate laboratory facilities, the remaining CSF samples were stored after the latex agglutination assay or bacteriologic tests had been performed. After laboratory personnel had been informed of the purpose of the surveillance, sterile tubes and epidemiologic forms were set up in 14 health districts within a radius of approximately 250 km of Niamey. The designated area represented approximately 35% of the whole population. Afterwards, the CSF samples were regularly collected by a CERMES vehicle, according to a precise timetable. Later, tubes and forms were also set up in all health regions of Niger, and the healthcare centers located beyond the limit of 250 km used any suitable opportunities to convey the CSF samples to CERMES. Thus, the microbiologic surveillance included >50% of the population. The implementation of this strategy and the use of the results were carried out in close collaboration with the national surveillance system.

CSF specimens were tested by PCR (amplification for 35 cycles) for the three main causative agents of acute bac-

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terial meningitis in Niger: *N. meningitidis* (10), *Streptococcus pneumoniae* (11), and *Haemophilus influenzae* (12). A second PCR was performed on specimens positive for *N. meningitidis* to identify serogroups A, B, C, and Y/W135, and finally, serogroups Y/W135 were differentiated from both Y and W135 alone by a confirmative PCR (10). In the same manner, specimens positive for *H. influenzae* were further tested for type b DNA.

From November 2002 to May 2003, which included the entire season of transmission, 1,651 CSF specimens collected within the national surveillance system were processed by using PCR; 1,239 (75%) specimens came from outside the capital, Niamey. Until mid-2002, most of these specimens would have been lost for microbiologic surveillance. A total of 778 specimens (47.1%) were positive: 661 for *N. meningitidis* (85%), 83 for *S. pneumoniae* (10.1%), and 34 for *H. influenzae* (4.4%). The results of CSF examinations, according to the month and region, are presented in Figures 1 and 2. Surveillance highlighted that the meningococcal serogroup *N. meningitidis* W135 accounted for 8.5% of all *N. meningitidis* and that W135 was found in most of the regions in Niger, although it did not cause epidemics. *N. meningitidis* serogroup W135 was rare or absent in regions where *N. meningitidis* serogroup A epidemics occurred (three serogroup W135 isolates among 368 *N. meningitidis* isolates in Zinder and no W135 among 79 *N. meningitidis* isolates in Maradi). With 284 specimens from Niamey, which had undergone both PCR and bacteriologic testing, we compared the results from the two methods. The results of both tests were in agreement for 231 specimens (81.3%); results for 182 of those specimens were negative. PCR found 25 positive samples for which bacteriologic tests were negative, and 8 more positive specimens on which bacteriologic testing could not be carried out because of contamination. Conversely, bacteriologic testing provided a diagnosis for six PCR-negative samples. However, the region of Niamey presented the lowest overall rate of confirmation of suspected bacterial meningitis so this comparison is less conclusive. The two diagnostic methods were also applied to 102 specimens sent on trans-isolate medium. As is frequently observed, a contamination problem occurred, and 32 samples (31.3%) were unsuitable for bacteriologic testing, but PCR identified a causative agent in 20 of these 32 cases. Of the remaining samples, for 46 (65.7%), bacteriologic testing and PCR results were concordant, whereas 19 samples negative by bacteriologic testing, tested positive by PCR. The opposite was observed for two specimens.

The overall proportion of specimens that tested positive for one of the three species of bacteria varied according to the period. From November to February, before the meningitis epidemic season, the positivity rate was low in all the regions. In March and April, during the epidemic season, the positivity rate remained low in the districts not undergoing epidemics, although the positivity rate reached 60.9% in the Zinder region, where epidemics due to *N. meningitidis* serogroup A occurred. Because of the uncertainty of the cold chain in remote areas, some specimens may have been stored without taking into account the temperature conditions, which might have affected the sensitivity of the PCR testing. An analysis of confirmation rates must also take into account that CSF samples were tested whether they were cloudy or purulent or not and that the currently used PCR focuses on three species only, although

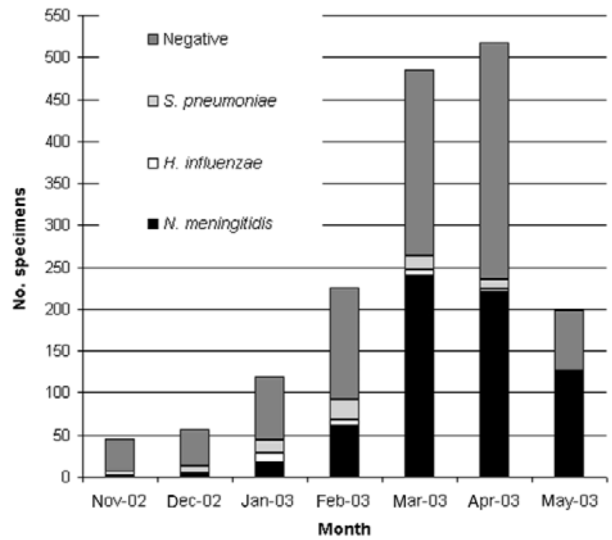

Figure 1. Results of polymerase chain reaction assay on cerebrospinal fluid specimens, November 2002–May 2003. *S, Streptococcus; H, Haemophilus; N, Neisseria*.

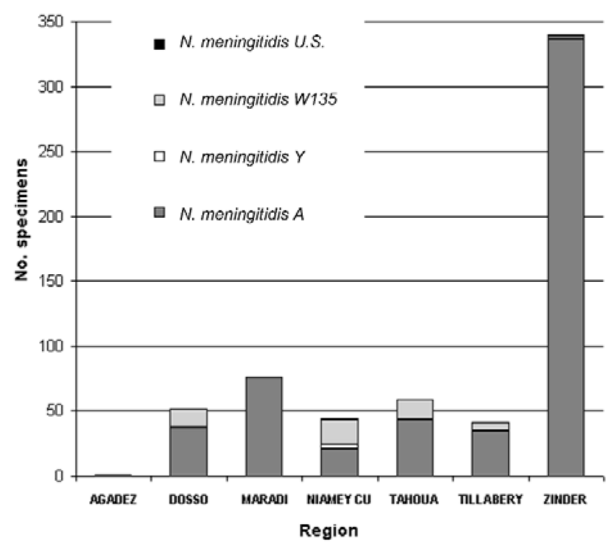

Figure 2. Distribution of serogroups of *Neisseria meningitidis* according to region, November 2002–May 2003. (*N. meningitidis* U.S. = unpredicted serogroup, i.e., not A, not B, not C, not Y and not W135.)

many other causes of meningitis exist. The proportion of negative results may also indicate that the national health services keep a vigilant watch over meningitis and that the diagnosis is widely used, perhaps excessively. The symptoms are not fully specific, and the predictive value of the clinical picture for meningococcal meningitis increases substantially during an epidemic, as shown by the results from Zinder.

In contrast to the culture method, which requires that laboratories receive live bacteria (quite restricting, given the fragility of *N. meningitidis*), PCR offers the substantial benefit of being able to be performed on dead bacterial cells that have been killed by either refrigeration or previous antimicrobial drug treatment. The simple way of storing and dispatching CSF samples collected in remote areas was convenient and realistic for the circumstances in Niger. Although the biologic confirmation by PCR is retrospective in our study and cannot be used for case management, this surveillance network compensates efficiently for the lack of functional laboratories at the local level outside of the capital. During the 2003 meningitis season, PCR assay allowed satisfactory monitoring of the causative agents of bacterial meningitis and of the involved meningococcal serogroups, which is important in adapting the most appropriate preventive strategy while serogroup W135 has the potential to cause epidemics in the countries of the meningitis belt.

In conclusion, to compensate for the severe shortage of laboratories outside the capital, the PCR assay proved to be a valuable tool for routine microbiologic surveillance of bacterial meningitis in Niger. The country has started implementing the Integrated Disease Surveillance and Response plan within which this microbiologic surveillance is fully integrated.

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Frequency of Revaccination against Smallpox

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Recent suggestions to revise guidelines that recommend extending the time for revaccination beyond 10 years may be based on insufficient and conflicting evidence of persistence of immunity (1,2). The evidence that cell-mediated immunity and neutralizing antibody persist after one vaccination is conflicting (2–5). This residual immunity is often low, and its protective activity in vivo is unclear. Similarly, in vivo reports of durable immunity to smallpox (1) were not sufficiently controlled, and short persistence of resistance to dermal challenge with the antigenically related vaccinia virus has been reported (3,6).

We confirmed the previous report (5) that the residual antivaccinia virus titers of serum samples from singly vaccinated adults are low (average 32) (Table). The titer of normal commercial immunoglobulin (Ig) (Panglobulin) (11 times concentrated sera) was 150 U/mL, which when calculated to include the 11-fold concentration, confirms the low residual titers.

The titers of the control, unvaccinated persons, averaged 14, raising questions about the importance and specificity of the residual antibody in vaccinated persons. We determined that the persistent neutralizing activity is mainly IgG antibody in serum from both single-vaccinated persons and ordinary commercial IgG, since sequential absorption with protein G beads and anti-IgG beads reduced the titers 80%. However, the neutralizing activity in unvaccinated control serum may not be mainly IgG antibody since neutralizing activity was reduced by an average of 48%, favoring nonspecific inhibitors. Studies of these nonspecific inhibitors and possible cross-immunizing antigens in the environment should be conducted to explain the occurrence of neutralizing activity in serum of unvaccinated persons.

To determine whether the low residual titers in sera from single vaccinated persons protected in vivo against a systemic infection, mice were pretreated subcutaneously with 1 mL of either 1) serum from a single-vaccinated study participant containing the low 10 U/mL neutralizing activity (patient A), 2) serum from a single-vaccinated person containing the higher 43 U/mL (patient E), or 3) normal commercial Ig containing 150 U/mL and challenged 24 hours later with one LD_{100} vaccinia virus, strain IHD-E, intraperitoneally. The 1 mL of serum injected into the mice is estimated to provide its original titer in the mouse. The lowest titer serum (10 U/mL) did not protect the mice against lethal systemic infection, whereas the highest titer serum (43 U/mL) and the commercial Ig (150 U/mL) protected 50% of the mice. Thus, the levels of residual antibody in vaccinated persons are either not protective or only partially protective in mice. Consistent with the reported protection by the higher levels of antibody, vaccinia

Table. Residual vaccinia virus neutralizing titers of serum from vaccinated and unvaccinated persons

Participant no.	Y after vaccination	Neutralizing titer
Single-vaccinated persons		
A	47	10
B	50	26
C	45	27
D	40	34
E	40	43
Ordinary commercial immunoglobulin (Panglobulin)		150
Unvaccinated persons		
H6	—[a]	<10
H9	—	<10
C2	—	<10
C4	—	<10
H1	—	10
H2	—	10
H13	—	10
C1	—	10
C3	—	10
H7	—	10
HL	—	20
H3	—	20
H4	—	20
H5	—	20
H10	—	20
H11	—	20
H12	—	20
C7	—	20
H8	—	30

[a]—, Not applicable.

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immune globulin (VIG), which contains 500 neutralizing U/mL, is effective under some conditions (7–10). As a positive control for protection in this animal model, 100 µg of the interferon inducer Poly I:CLC protected 100% of the mice. Undetermined and requiring study is whether active immunity might be protective through an anamnestic response. The animal models of poxvirus infection have been used to evaluate immunity, but no generally established laboratory surrogate exists for immunity to smallpox virus itself. Persistence of effective humoral immunity after a single vaccination and its ability to effectively protect in vivo remain questionable.

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Commentaries

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

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Asymptomatic Severe Acute Respiratory Syndrome–associated Coronavirus Infection

To the Editor: An outbreak of severe acute respiratory syndrome (SARS) began in Hong Kong in March 2003. As of May 29, 2003, a total of 1,732 cases were confirmed; 381 case-patients were healthcare workers and medical students. Clinical features, treatment protocols, and outcomes have been previously reported by various local experts (1–3). The etiologic agent is a SARS-associated coronavirus (SARS-CoV) (1). However, no asymptomatic case of SARS-CoV infection has been previously reported (4). In addition, in Hong Kong, blood donors have not shown any detectable antibody to SARS-CoV (1). We report a case of possible asymptomatic SARS-CoV infection in Hong Kong.

The case-patient is a registered nurse working in Princess Margaret Hospital, the major infectious diseases hospital that treated >600 SARS patients in Hong Kong. Within this hospital, >800 frontline staff members have participated in direct care of SARS patients, and SARS developed in 62 of these staff members. All healthcare workers working in SARS wards followed the same infection control measures, wearing a N-95 respirator, eye shield, disposable cap, water-resistant gown, and gloves. Gowns and equipment were removed before the staff left the SARS wards.

We performed serologic testing of the first 101 healthcare workers (doctors, nurses, healthcare assistants) who worked in the SARS wards but in whom SARS did not develop. The serologic testing was performed 7–8 weeks after the healthcare workers were first exposed to SARS patients.

We identified a nurse who was asymptomatic for SARS-CoV infection, worked in the SARS ward since the disease outbreak, and used full infection control procedures as recommended by the World Health Organization (WHO). The nurse performed procedures, including nasopharyngeal aspiration, handling of fecal matter, and oral feeding of SARS patients. SARS developed in six colleagues who worked in the same ward. She had unprotected exposure to a colleague who contracted SARS and required hospitalization. Serologic testing for SARS-CoV antibody was performed in the microbiology laboratory of Princess Margaret Hospital on week 8 of the nurse's SARS ward duty. The result of the test was positive by enzyme-linked immunosorbent assay. The test was repeated by the Government Virus Unit of the Department of Health, one of the reference laboratories in Hong Kong. The second test also showed a positive result with an antibody titer of 400 by immunofluorescence assay (normal: <25). We performed another serologic test on week 10 of her SARS ward duty; the result was again positive. The nurse was interviewed by two physicians and questioned about her health condition since February 2003. She did not report any symptoms typical of SARS, such as fever, chills, rigors, malaise, myalgia, cough, dyspnea, and diarrhea (1,3) during and after her SARS ward duty. She did have a mild, short-term headache, which she has had periodically for many years. She did not take sick leave since February 2003. She did not record any rise in body temperature >37°C and had a leukocyte count of 5.9×10^9/L and a lymphocyte count of 1.6×10^9/L. Results of liver and renal function tests were all normal. Reverse transcription-polymerase chain reaction results for SARS-CoV in stool, urine, throat, and nasal swabs collected during weeks 10 and 14 of her SARS ward duty were all negative. No abnormal radiologic change was identified in the lungs. She lived with four family members and had close contact with them. None of her family members contracted SARS, and all showed a negative result in the serologic testing for SARS-CoV.

We think that asymptomatic and subclinical infection of SARS-CoV exists and can result in seroconversion; however, this kind of asymptomatic seroconversion is probably uncommon. Why a person infected with SARS-CoV did not have typical symptoms, and the infectivity of an asymptomatic person is unknown. A person's genetic makeup may determine susceptibility to SARS-CoV and the final clinical outcome. We agree with Seto et al. (5) that recall bias is a concern. However, recall bias probably had little effect since the events took place recently. Moreover, the hospitalization of the nurse's infected colleague would have made her more alert and aware of symptoms of the illness.

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Hepatitis C Antibodies among Blood Donors, Senegal, 2001

To the Editor: Prevalence of chronic hepatitis C virus (HCV) among blood donors has been assessed in a few West African countries; most recent estimates range from 1.1% to 6.7% (1–4). A recent meta-analysis of studies, including a confirmation test, yielded an average prevalence of HCV infection of 3.0% (5). Until 2001, no systematic screening of HCV infection occurred among blood donors in Senegal, and blood donation legislation is still pending. We report an assessment of the proportion of blood donors from the Hôpital Principal de Dakar who had HCV antibodies in 2001.

Blood donors were all volunteers, recruited independently from the hospitalized patients and registered in a local donors association. We screened for risk factors for bloodborne infections in potential donors through a clinical examination and a confidential questionnaire. Persons with a history of jaundice or a risk behavior were excluded. Serum samples collected from blood donors from June to December 2001 were screened for HCV antibodies by a third-generation enzyme immunoassay (EIA) (HCV Murex 4.0; Abbott Laboratories, Abbott, IL). Confirmation was performed by a recombinant-immunoblot assay (INNO-LIA HCV Ab III update; [Innogenetics, Gent, Belgium]). HCV RNA was detected by a qualitative reverse transcription–polymerase chain reaction (Roche Amplicor HCV test [Hoffman-LaRoche, Basel, Switzerland]). Genotype was determined by the INNO-LiPA HCV II assay (Innogenetics). Presence of hepatitis B surface antigen (HbsAg) and alanine-aminotransferase (ALAT) level are routinely assessed, as well as HIV and human T-lymphotropic virus type l infection.

The age of the 1,081 donors ranged from 18 years to 61 years (mean 35.6 years), and 81% were men. First-time donors accounted for 31% and were younger than repeat donors (mean 30.5 years vs. 37.8 years; $p < 10^{-4}$). EIA HCV antibodies were found in 18 donors (1.6%). Immunoblot assay was positive for nine, yielding an overall prevalence of 0.8% (exact 95% confidence interval 0.4% to 1.5%). Eight of the nine were repeat donors, but the difference in prevalence compared with first-time donors did not reach statistical significance (1.1% vs. 0.3%). HCV-infected donors tended to be older than uninfected donors (mean 42.3 years vs. 35.5 years, median 46.7 years vs. 34.6 years, Mann-Whitney test p = 0.04), and the trend with age was significant (18–29 years 0.3%; 30–39 years 0.6%; 40–49 years 1.5%; $\geq$50 years 1.8%; chi-square trend = 4.39; p = 0.03). ALAT levels of infected study participants were in the normal range (17–55 IU). One participant had an ALAT level above normal. Genotype 2ac has been identified on line immunoassay–positive samples (three samples not tested). HBsAg was detected in 13% of the new donors. No co-infection with HCV and hepatitis B virus was found.

The prevalence of HCV antibodies in blood donors in Dakar in 2001 appears to be one of the lowest in West Africa, close to published estimates for Mauritania and Benin (1.1% and 1.4%, respectively) and lower than in other West African countries such as Ghana or Guinea, where prevalence ranges from 2.8% to 6.7% (1–4). This finding is in keeping with results of a hospital case-control study on HCV infection and liver cirrhosis or cancer, conducted in 1995 in Dakar. While that study did not identify HCV infection in 73 controls, 2 of 73 case-patients (2.7%) had HCV antibodies (6). Conversely, high HCV prevalence was found in groups at risk: antibodies were present in 12 of 15 hemodialysis patients, and HCV RNA was found in 6 of the 12 HVC antibody-positive patients (genotype 2ac, the same as in our study); 7% of a cohort of 58 HIV-1 patients receiving highly active antiretroviral therapy had a positive HCV serologic result (7,8).

In the urban setting of Dakar, HCV infection seems still to be confined to groups at risk. The contribution of HCV to chronic liver diseases has not been yet demonstrated. Approximately 15,000 blood donations are annually made in Dakar. A systematic screening of HCV antibodies in blood donors could prevent, on average, 120 bloodborne HCV infections each year. Given these data and the price of EIA and LIA, the screening cost per HCV-positive sample identified, and infection subsequently averted, is approximately 200,300 CFA (U.S.$305). This estimate is low since it includes only the marginal cost of the reagent kits. This screening cost could be reduced by discarding blood units that test positive after only one enzyme-linked immunosorbent assay (156,000 CFA or U.S.$237), at the price of nearly 3% of blood units wrongly discarded. France has demonstrated that this strategy has the best cost-effectiveness ratio, as long as the prevalence remains below 8% (9). This cost compares favorably with the cost per HIV infection averted through improvement of blood safety (range U.S.$20–U.S.$1,000), assessed in some highly HIV-prevalent southern African countries (Tanzania, Zambia, Zimbabwe) (10). The HCV-positive discarded blood units will be added to the blood units testing positive for hepatitis B surface (13%), HIV, and HTLV, which accounted for nearly one third of all donations in 2001. These findings argue in favor of maintaining a roster of regular, seronegative donors to save numbers of blood units.

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Prosthetic Valve Endocarditis due to *Kytococcus schroeteri*

To the Editor: Bacteria belonging to the former genus *Micrococcus*, the so-called micrococci, are usually regarded as contaminants from skin and mucous membranes. Nevertheless, micrococci have been reported as emerging pathogens in immunocompromised patients and have been described in severe infections (1–4). We describe what is, to our knowledge, the first case of prosthetic valve endocarditis caused by the newly described micrococcal species, *Kytococcus schroeteri*. Accurate identification of this species is of particular importance as kytococci—in contrast to other micrococcal species—are frequently resistant to penicillin and oxacillin (5).

A 34-year-old woman was admitted to the hospital with acute, severe aortic regurgitation, attributable to a dissection of both the ascending and descending aorta, which extended into the supraaortic and iliac arteries. Immediate surgical intervention was performed by implantation of an aortic arch (St. Jude Medical Inc., St. Paul, MN) conduit and reimplantation of the supraaortic arteries. Ten weeks later, the patient was admitted to the hospital because of fever of 39°C. Laboratory studies showed a leukocyte count of 15.3 x 10^9/L with 87% neutrophils and elevated C-reactive protein (180 mg/L). Transesophageal echocardiography and computed tomography suggested an abscess next to the prosthesis and showed vegetations on the prosthetic valve, which suggested endocarditis. Blood cultures yielded gram-positive cocci on four separate occasions during an 11-day period. Treatment, performed according to the antimicrobial susceptibilities of the isolates, consisted of vancomycin, gentamicin, and rifampin for 21 days. Within 1 week, the fever resolved and the leukocyte count returned to normal. Four days after antimicrobial therapy was initiated, right-sided hemiparesis and aphasia, thought to be due to an embolic cerebral stroke, developed. After those events, the aortic arch prosthesis was replaced without further complications.

Blood culture specimens were injected into BACTEC Plus culture vials for aerobic and anaerobic cultures and processed in BACTEC 9240 blood culture system (Becton Dickinson, Cockeysville, MD). Growth was detected in four different aerobic blood cultures after incubation of 3 to 5 days. Aerobic subcultures on Columbia agar supplemented with 5% sheep blood showed tiny, muddy-yellow colonies without hemolysis after 24 h of incubation. After 48 h, the size of colonies increased, a feature typical of *K. sedentarius*, which is known to grow slightly more slowly than other members of the former *Micrococcus* genus. No or very weak reactions were found after 24 h incubation when the ID32 STAPH ATB gallery (bioMérieux Vitek, Hazelwood, MO) was used. After 48 h, the reactions with this gallery resembled those of *M. luteus* or *M. lylae.* The probability of identification was indicated as 99.0% (*M. luteus*, T index of 0.77) for the profile 000003000 and 51.8% (*M. lylae*, T index of 0.98) and 47.2% (*M. luteus*, T index of 0.93), respectively, for the profile 000001000. When the ID-GPC card (VITEK 2, bioMérieux Vitek) was used, a poor selectivity was observed (*M. luteus*, T index 0.95; *Kocuria rosea*, T index 0.84). All isolates were resistant to oxacillin, penicillin, fosfomycin, ampicillin, and erythromycin and susceptible to vancomycin, teicoplanin, gentamicin, netilmicin, chloramphenicol, imipenem, rifampin, tetracycline, amoxicillin/clavulanate, and ciprofloxacin, as determined by disk diffusion method performed on Mueller-Hinton agar.

When arbitrarily primed-polymerase chain reaction with prolonged ramp times (6) was used, isolates were shown to be clonal, representing one strain (DSM 13884^T). Since colony formations, resistance pattern, and growth rate of this strain did not correspond with the species identification, as obtained by automated systems, further phenotypic and molecular studies were conducted, confirming the micrococcal nature of this unknown strain and justifying the classification as a distinct species, *Kytococcus schroeteri* sp. nov. (7).

In addition to the *Micrococcus* genus, bacteria belonging to the former genus *Micrococcus* were recently divided into the genera *Kocuria*, *Nesterenkonia*, *Kytococcus*, and *Dermacoccus,* followed by rearrangement into two families (*Micrococcaceae, Dermatophilaceae*) of the suborder *Micrococcineae* (5).

The traditional identification of the micrococci is based on their susceptibility to lysozyme and bacitracin and their resistance to lysostaphin and nitrofurantoin, in contrast to staphylococci, which display the opposite pattern. In automated identification systems, micrococci are included only in a limited manner. A prospective study showed an overall accuracy of results of 61.0% concerning *Micrococcus* species when the STAPH-IDENT strip (bioMérieux) was compared with conventional identification methods (8).

Micrococcal species are ubiquitous inhabitants of the human skin and mucous membranes and are usually disregarded as contaminants in clinical specimens. Yet, various severe infections such as arthritis, central nervous system infection, pneumonia, peritonitis, hepatic abscess, endocarditis, and nosocomial blood stream infections have been documented (1,3,4,9). Since early reports of endocarditis caused by gram-positive cocci that appear in tetrads and packets often did not reliably differentiate between micrococci and phenotypically similar microorganisms, such as coagulase-negative staphylococci, the frequency of micrococcal endocarditis is difficult to ascertain and might be underestimated. However, several cases of endocarditis attributable to *M. lylae*, *M. luteus*, *K. sedentarius,* and unspecified micrococci have been reported (1).

Regarding micrococci, data on antimicrobial susceptibilities are rare, and often the species affiliation remains unclear. In contrast to most micrococcal isolates, *K. sedentarius* isolates, as well as those reported here, are resistant to penicillin G and oxacillin. In the patient we describe, therapy was performed with vancomycin, gentamicin, and rifampin, resulting in bacteriologic eradication and clinical cure. However, a generally accepted therapeutic regime for severe infections with kytococcal species has not yet been defined. Concerning micrococci other than kytococci, a combination of rifampin with ampicillin has been effective (3). Successful treatment has also been achieved with vancomycin, clindamycin, penicillin, gentamicin, or a combination of these agents. Overall, rifampin shows the highest activity against all micrococcal species (10).

This report is the first case of *K. schroeteri* causing endocarditis on an artificial heart valve. The repeated recovery of this species from blood cultures strongly suggests a pathogenic role. We conclude that isolation of micrococci from blood specimens cannot always be disregarded as etiologically irrelevant. Results performed by automated identification systems should be interpreted with caution if micrococci are involved.

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When Is a Reservoir Not a Reservoir?

To the Editor: Some 80% of parasitic infections of humans are zoonoses (1). These infections are caused by multihost parasites for which the reservoir of infection depends on hosts other than *Homo sapiens*. But what is a reservoir of infection?

Haydon et al. (2) proposed a new series of definitions in connection with multihost pathogens, in which a target host is the host of interest in a particular context. The reservoir of infection included, for these authors, all hosts, whether incidental or not, that are epidemiologically connected to (i.e., contribute to transmission to) the target host.

The availability of three terms—reservoir, reservoir of infection, and reservoir host—frequently used interchangeably, leads to confusion. This confusion is, in part, what prompted me (3) to slightly redefine a reservoir (of infection) as an ecologic system in which an infectious agent survives indefinitely. Such a system includes all the component host populations, including that of any intermediate host or vector, in the context of any environmental component, and any quantitative requisite such as critical community size, which is required to maintain the agent indefinitely.

Vertebrate hosts that form an essential part of the system are reservoir hosts, though whether a whale or a fish is the reservoir host of *Anisakis* species can be a matter of debate. A host that becomes infected, but is not required for the maintenance of the population of a pathogen, can usefully be called an incidental host. (Accidental host is frequently used, but this is arguably a teleological term and therefore undesirable.) For incidental hosts that transmit pathogens from a reservoir to another incidental host, analogous to a pipe leading from a water reservoir, Garnham (4) coined the useful term "liaison host."

Haydon et al. dismiss my definition on two grounds. First, I exclude liaison hosts from the reservoir. This distinction is valid and could be argued either way, but I suggest that the pipes leading from a reservoir do not form part of the reservoir and that it is both conceptually and practically important to distinguish liaison hosts from reservoir hosts. The second objection is that many pathogens depend on the presence of several host species, at any given stage in the life history, for their maintenance. This concept is clearly considered in my article: together, such hosts collectively constitute part of the reservoir system, though no single one may be the reservoir host in its own right.

In good scientific English, each term should have a precise definition, and synonyms should be avoided. The Oxford English Dictionary (OED) (5) definitions of reservoir generally refer to a place or container used for the collection and storage of water, other fluids, or even solid material.

The OED definition of reservoir as a medical term is. "A population which is chronically infested with the causative agent of a disease and can infect other populations." While one might argue with the terms chronically, infested, and the infection of populations, this definition captures the usual sense in which reservoir host is used.

The quotations given in OED are more helpful. The earliest one given for reservoir in a medical context is from 1937, "For the continuous existence of a disease there must be some reservoir of infection… The most important reservoirs of infection are human or animal cases or carriers. Plants may be the reservoir of infection in some of the mycoses." However, according to OED, the compound term "reservoir host" was used earlier, in 1913, "The monkey is most probably the normal reservoir host [for *Physaloptera mordens*]."

The main conceptual difference between the proposal of Haydon et al. and my own is that mine is more generalized: for a given pathogen in a given place, there is a single reservoir. The proposal of Haydon et al. is more limited to practical considerations: the reservoir for one target host may not be the same as that for another target host in the same place.

The most important contribution of these two publications is that they raise an issue that has confused the literature for many years. Parasitologists, virologists, and bacteriologists should agree on a consensus set of terms for the ecologic description of multihost systems. When we all agree on what we are talking about, we will understand each other better.

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Invasive *Mycobacterium marinum* Infections

To the Editor: *Mycobacterium marinum* infections, commonly known as fish tank granuloma, produce nodular or ulcerating skin lesions on the extremities of healthy hosts. Delay of diagnosis is common, and invasion into deeper structures such as synovia, bursae, and bone occurs in approximately one third of reported case-patients (1).

A 49-year-old man with diabetes, who had received a kidney transplant from a living relative 8 years previously, sought treatment after 5 months of worsening swelling and tenderness of the left elbow. Of note, he had injured his left ring finger while cleaning barnacles from a piling 5 years previously and had contracted a secondary infection that never completely healed despite three courses of antimicrobial drugs and surgical debridement. Physical examination showed marked swelling, tenderness, and warmth of the left elbow, as well as of the left ring finger, which was erythematous. Sterile aspiration of the olecranon bursa showed 7,500 leukocytes (62% lymphocytes) and 141,000 erythrocytes. Results of Gram stain and routine cultures were negative. Magnetic resonance imaging of the left arm showed soft tissue edema of the olecranon bursa and the left fourth flexor digitorum longus tendon, and no osteomyelitis. Three weeks later, olecranon bursa aspirate fluid cultures incubated on chocolate agar and 7H11 plates at 31°C, as well as on algae slant, and mycobacterial growth indicator tubes incubated at 37°C grew *M.ycobacterium marinum*. The isolate was susceptible to most agents but showed intermediate susceptiblity to ciprofloxacin (MIC 2 µg/mL) and was resistant to ampicillin/clavulanate and erythromycin (MIC 8 µg/mL and 32 µg/mL, respectively). A treatment regimen of rifampin and ethambutol was begun, and the patient showed a dramatic improvement in the ensuing several weeks. The patient has completed 9 of 11.4 planned months of therapy and continues to do well, with frequent office visits.

Case reports from English language MEDLINE articles since 1966 under the subject heading *Mycobacterium marinum* were cross-referenced with articles containing the following text words: disseminated, osteomyelitis, arthritis, synovitis, and bursitis. Ten case reports were identified, and a hand search through pertinent articles' references yielded 13 additional reports. A total of 35 cases of invasive *M. marinum* disease were then reviewed, according to patient age and sex, symptoms, source of infection, immune impairment, time to diagnosis, and type as well as duration of therapy (2–24) (see online Table at http://www.cdc.gov/ ncidod/EID/vol9no11/03-0192.htm#table).

Most cases occurred in previously healthy adults. The average age was 43 years; 24 (69%) were men; 21 (60%) had tenosynovitis; 6 (17%) had septic arthritis; and 13 (37%) had osteomyelitis. In three patients (9%), either a bone marrow or blood culture positive for *M. marinum* was obtained; all three patients showed marked systemic immunocompromise. Multiple skin lesions were seen in 23% of cases; half of these patients showed clear evidence of deeper infection. Some patients had more than one manifestation of invasive disease. Immunologic impairment was a frequent component of invasive *M. marinum* infections: 14 (40%) of case-patients received a steroid injection at the site of infection, and 9 (26%) were receiving systemic steroids for various indications. An additional 4 (11%) case-patients were in an immunocompromised state from other sources such as chemotherapy or AIDS. Delayed diagnosis was also a prominent finding: The average time to diagnosis was 17 months from symptom onset. The treatment course was prolonged and aggressive: The average treatment duration was 11.4 months in the 20 reports in which a definitive duration was given. Surgery was undertaken in 69% of the cases. The treatment regimen used varied considerably, although 30 (88%) of the 34 patients who took antimycobacterial medications received combination therapy. Rifampin (76%) and ethambutol (68%) were the predominant agents.

While *M. marinum* infections usually arise from aquatic trauma in healthy hosts, delayed diagnosis and immune suppression contribute to the pathogenesis of invasive infection. Tenosynovitis is the most common manifestation of deep invasion, although septic arthritis and osteomyelitis are well described. Disseminated skin lesions can accompany deeper invasion but may be seen in isolation as well. Bone marrow invasion and bacteremia are rare and have been seen only in profoundly immunocompromised patients.

Although the rarity of the condition makes estimating its incidence difficult, the number of case reports per year has remained stable for the last 30 years. However, the high frequency of delayed diagnosis in cases of invasive *M. marinum* disease underscores the importance of maintaining a high level of suspicion for this condition, especially in patients who have evidence of previous aquatic trauma or refractory soft tissue infections. Further, since immunosupression was common in cases of invasive disease, local steroid injections should be avoided in patients with soft tissue infection after aquatic trauma at least until *M. marinum* infection is ruled out by acid-fast staining or mycobacterial culture of biopsy specimens or fluids.

Once invasive *M. marinum* disease was diagnosed, patients with invasive disease were treated for an average of 11.4 months, three times longer than the typical course for *M. marinum* superficial infections (1). Rifampin and ethambutol were used most often in invasive infections, although many therapeutic choices exist. In a study of 61 clinical isolates, rifamycins and clarithromycin were the most potent, with the lowest MICs, and resistance was uncommon. Doxycycline, ethambutol, and minocycline all showed higher MICs but were still effective (1). A different group tested 11 agents against 37 clinical isolates and found that trimethoprim/sulfamethoxazole was the most potent agent, but 92% of isolates were susceptible. Clarithromycin and minocycline, by contrast, showed susceptibility rates approaching 100% and retained similar potency (25). This study reported an MIC_{50} for most quinolones of 4 µg/mL or higher, although in a different study, 100% of *M. marinum* isolates were susceptible to gatifloxacin (26). Approximately three fourths of isolates in this latter study were susceptible to ciprofloxacin and levofloxacin. Among newer antibiotics tested against *M. marinum* in this series, only linezolid showed much promise (26). On the basis of the sparse data correlating susceptibility testing results to clinical response, and the relative infrequency of resistance, recent guidelines suggest foregoing susceptibility testing in *M. marinum* infections unless the infection does not respond to treatment (27). Most cases of invasive *M. marinum* infection require surgical debridement, 69% in this series. This approach seems particularly appropriate in immunocompromised patients, those with tenosynovitis, or those for whom medical therapy fails.

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Rickettsialpox in Turkey

To the Editor: Rickettsialpox is often described as a chickenpox-like disease and is caused by *Rickettsia akari*, a spotted fever group *Rickettsia* that is transmitted to humans by the bite of mites (*Liponyssoides sanguineus*). Although the mite host (typically a mouse) is widely distributed in cities, the disease is infrequently diagnosed. It is typically characterized in patients by the appearance of a primary eschar at the site of a mite bite followed by fever, headache, and development of a papulovesicular rash. Symptoms normally appear 9–14 days after the mite bite and are often unnoticed by the affected person. In documented rickettsialpox cases, the presence of a papule that ulcerates and becomes a scar approximately 0.5–3.0 cm in diameter is reported (1–3). Three to 7 days later, symptoms are more pronounced, with patients experiencing the sudden onset of chills, fever, and headache followed by myalgia and the appearence of generalized vesicular skin rashes. Less frequently, photophobia, conjunctival injection, cough, generalized lymphadenopathy, and vomiting are reported.

The first well-described clinical case of rickettsialpox was documented in New York City in 1946 (1). Historically, most documented rickettsialpox cases have occurred in large metropolitan areas of the United States (2), where the causative agent, *R. akari*, circulates primarily between the house mouse (*Mus musculus*) and its mite (*Liponyssoides sanguineus*). Recently, rickettsialpox cases have been reported from Croatia, Ukraine, South Africa, Korea, and North Carolina (3,4). *R. akari* was isolated from the blood of a patient suspected of having Mediterranean spotted fever rather than rickettsialpox; this was the first human isolate of *R. akari* reported in >40 years (4). Recent reports of a rickettsialpox case in North Carolina (3), *R. akari* seropositivity found in HIV-positive intravenous drug users in the inner city of Baltimore, Maryland (5), and in Central and East Harlem, New York City (6), as well as rickettsialpox cutaneous eruption in an HIV patient in New York (7), indicate that *R. akari* rickettsiosis is more common than previously thought and presents the risk of sporadic outbreaks worldwide.

We describe the clinical presentation of rickettsialpox in a 9-year-old boy from Nevpehir, located in the middle region of Turkey. Previously, a report from the Antalya area of Turkey described the prevalence of serum immunoglobulin (Ig) G antibodies in humans directed against *R. conorii* (spotted fever group *Rickettsia*) (8); however, rickettsialpox was not reported in Turkey. This report of what we believe to be the first described rickettsialpox case from Turkey further extends the recognized geographic distribution of *R. akari*.

A 9-year-old boy was admitted to the Kayseri hospital with fever >39°C and generalized papulovesicular exanthema. One week before admission, fever, profuse sweating, headache, and dysuria were present. On admission, physical examination indicated generalized vesicular, bullouse, and papular exanthema involving the lips and oral cavity. Notable pathologic findings at admission included a black eschar on the boy's penis, bilateral prominent conjunctival ejection, and bilateral lower pulmonary rales. The leukocyte count was 13,300/mm^3, hemoglobin was 14.49 mg/dL, and the platelet count was 544,000/mm^3. Serum electrolytes and blood urea nitrogen levels and results of coagulation study and urine analysis were normal. Routine blood cultures taken 24 hours postadmission were sterile. Specific antibodies (IgG; IgM) against *Varicella* were not detected in serum samples (Duzen Laboratories, Ankara, Turkey). Additionally, the patient reported mice on the family's farm.

A diagnosis of rickettsialpox was made and doxycycline treatment (200 mg/kg) was initiated. The patient serum sample was tested by indirect immunofluorescence assay (IFA) for IgG and IgM antibodies reactive with *R. akari* (Kaplan strain), *R. typhi* (Wilmington), *R. rickettsii* (Sheila Smith), and *R. conorii* (Malish 7). Serum IgG titers of 1/1280 and IgM of 1/40 to *R. akari* were detected and confirmed through cross-adsorption with rickettsial antigens (*R. rickettsii*, *R. conorii*) (9,10). Higher reciprocal titers were obtained against *R. akari* antigens than against *R. rickettsii* and *R. conorii* antigens (reciprocol titers of 1,024 vs. 512 and 512, respectively). We observed a difference in reduction in antibody titers against *R. akari* after adsorption with *R. akari* (Kaplan) (<16), *R. rickettsii* (256), and *R. conorii* (256). Antibodies against *R. typhi* were not detected. The IFA result confirmed the clinical diagnosis of *R. akari* infection. After 2 days of doxycycline treatment, the patient was afebrile, and the rickettsialpox infection resolved without scars or complications.

In summary, we present a case in which the presence of an eschar on the patient's penis, the failure of lesions to appear in crops, the sparsity of lesions, and mice on the family's farm led to a diagnosis of rickettsialpox, which was confirmed by cross-adsorption serologic findings. This case indicates that rickettsialpox is an emerging infectious disease in Turkey. We recommend further studies to define the prevalence of *R. akari* and the worldwide distribution of rickettsialpox.

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Human Granulocytic Ehrlichiosis in Estonia

To the Editor: We report a case of a 24-year-old woman living in a rural area of Estonia who had weakness, chills, and diarrhea on May 10, 2002. On day 5 of the illness, she was admitted to the Department of Infectious Diseases, University of Tartu, with high fever (38.5°C) and muscle pains throughout her body. Examination showed mild jaundice, painful and enlarged liver, and inability to move. Throat was erythematous, and enlarged lymph nodes were palpable on the neck.

Laboratory findings included the following: leukopenia 2.04×10^9/L; erythrocytes 4.08×10^{12}/L; hemoglobin 130 g/L; thrombocytopenia 36×10^9/L; eosinophils 0%; basophils 1.0%; monocytes 7.5%; lymphocytes 38.0%; neutrophils 51.0%; reactive lymphcytes 2.0%; plasma cells 0.5%; C-reactive protein 38 mg/L (normal <5 mg/L); bilirubin 95 μmol/L (normal <17 μmol/L); aspartate aminotransferase 121 U/L (normal <31 U/L); alanine aminotransferase 108 UL (normal <31 U/L); and alcaline phosphatase 200 U/L (normal 35–104 U/L). Ehrlichiosis was suspected by clinical symptoms and leukopenia, thrombocytopenia, and elevated transaminases.

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne disease described for the first time in 1994 in the United States (1). The first European case of HGE was reported in Slovenia in 1996 (2). Infection with *Ehrlichia phagocytophila,* the agent of HGE, occurs in areas endemic for *Borrelia burgdorferi* (3). In Estonia, Lyme borrreliosis is frequently diagnosed in humans but the occurrence of ehrlichiosis has not been established for this region, despite our having found some seropositive results in Lyme borrreliosis patients (4).

This case of ehrlichiosis is the first diagnosed in Estonia. The initial diagnosis was based on a typical clinical spectrum of symptoms and clinical laboratory findings, which are relatively nonspecific, making the diagnosis problematic (5). Polymerase chain reaction results for *Ehrlichia* were negative, and we did not find morula in the blood smear. Indirect immunofluorescence assay (IFA, MRL Diagnostics, Cypress, CA) was used as a confirmatory serologic test. However, results of this assay are often negative during the initial phase of the disease (5,6). On day 7 of illness, the serologic results for immunoglobulin (Ig) M type antibodies to *Ehrlichia* were positive (1:20) and negative for IgG. The diagnosis of ehrlichiosis was established, and therapy with doxycycline was started. After 4 days, the patient became afebrile, and on day 6 she left for home. One month later, the titers of both types of antibodies to *Ehrlichia* were increased: IgM titer was 1:160 and IgG titer was 1:128; 6 months later, IgM antibodies were negative, and the IgG titer remained unchanged.

Our patient had a typical spectrum of clinical and laboratory changes to *Ehrlichia,* but not very specific findings of infection with *E. phagocytophila*. The results of IFA, i.e., IgM antibodies in the beginning of the disease and increasing titer of IgG antibodies during the course of the disease, confirmed the diagnosis. Granulocytic ehrlichiosis should be considered in patients with tick-associated fever.

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Atlas of Travel Medicine and Health

Jane Chiodini
and Lorna Boyne, authors
BC Decker, Hamilton, Ontario, Canada, 2002, ISBN: 1-55009-189-1, Pages: 160, Price: $59.95

Travel medicine is a field that requires in-depth knowledge about disease epidemiology. Whether providing pretravel evaluations or posttravel consultations, the travel medicine specialist must be up to date on disease epidemiology. Pretravel evaluations require counseling the traveler to avoid risks from diseases specific to the itinerary. Posttravel consultations demand recognition of possible diseases on the basis of areas visited as well as specific exposures. A pictorial depiction of disease distribution can be a useful tool in determining risks of diseases to the traveler and can complement the existing resources for clinicians.

Atlas of Travel Medicine and Health, a book and accompanying CD-ROM by J. Chiodini and L. Boyne, contains basic information that can be used to provide pretravel advice. Its major sources are the World Health Organization and U.K. Department of Health and the Public Health Laboratory Service guidelines. The book is organized into three sections. Section One describes general risks and precautions, such as food and water, animal contact, vector-borne diseases, fresh water exposure, and blood and body fluid contact. Section Two contains descriptions and maps of some diseases that may be encountered by travelers. Section Three contains country-specific information about malaria, immunizations, and other health considerations for the more popular destinations.

The format is organized, the content is basic, and the book is easy to read. Although the intended audience is healthcare professionals, the information can be easily used by the traveler. Some maps have incorporated the topography to indicate specific malaria risk, depending on elevation, which is a nice feature.

Because Section Three only focuses on popular destinations, many countries with malaria risk are not included. The section can be improved with consistent use of color, a more detailed key, and by adding major tourist centers and cities. Although the authors do not intend to cover an exhaustive list of diseases or countries, another important improvement would be the inclusion of all countries infected with or endemic for yellow fever. Diseases such as leishmaniasis, Lyme disease, and acute mountain sickness deserve mention in Section Two but are not currently discussed. Finally, the CD-ROM is generally easy to use, but Section Two on the CD-ROM could be improved by changing the orientation of the maps.

We live in a world of movement. The mobility of populations globally leads to the potential spread of infectious diseases and to the rapid change of disease distribution as demonstrated by outbreaks of dengue, meningococcal disease, and severe acute respiratory syndrome (SARS). An ideal atlas for travel medicine should be able to reflect recent outbreaks in addition to the general distribution of diseases. To do so would require frequent updates of the atlas. Currently, the Atlas of Travel Medicine and Health is suitable for healthcare professionals and travelers who desire brief summaries on some travel-related illnesses, but it must be used in conjunction with more comprehensive references.

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Instructions for Infectious Disease Authors

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.

Conference Summary

Northern Ireland Food Safety Night

A meeting on food safety entitled “Food Safety: Is It All ‘“Pie-in-the-Sky?’“was held on June 19, 2003, in Belfast, Northern Ireland. Because Northern Ireland has a largely agrarian economy with a strong agricultural food sector, highlighting the importance of safe food production was timely. The meeting was organized by the Northern Ireland Microbiology Discussion Group (NIMDG) and was well attended by several local stakeholders in food safety, including representatives from hospital and public health microbiology laboratories, the Department of Agriculture and Rural Development of Northern Ireland, academia, and the food industry. The group heard presentations from Hugh Pennington, Department of Medical Microbiology, University of Aberdeen, Aberdeen, United Kingdom, and. Mike Kelly, Head of Food Safety and Environmental Health, British Airways.

Professor Pennington examined risk assessment in food safety, and discussed the approach to risk assessment of the oil and rail industries, and compared risk assessment between these industries and the food industry. He emphasized that, although science is important at addressing fundamental issues, an important emerging strategy is the translation of scientific findings into everyday practice to ensure that food safety is maintained. The attendees concluded that a greater interaction between microbiologists and psychologists should be encouraged, to add value to the science and ensure tangible benefits in reducing the incidence of foodborne illnesses. The group also learned that differences exist in the incidence of foodborne illnesses between Northern Ireland and Great Britain (England and Wales/Scotland), particularly with *Campylobacter* and *Salmonella* infections, and explored possible reasons for such differences, including climate, lack of consumption of unpasteurized milk, and the social custom of having foods “well done.”

Mr. Kelly described 42 documented cases of foodborne infections found in the literature that involved contaminated foods on aircraft and detailed, in practical terms, how the concept of a hazard analysis critical control point (HACCP) has been used successfully by airline caterers to reduce such infections to a rare occurrence.

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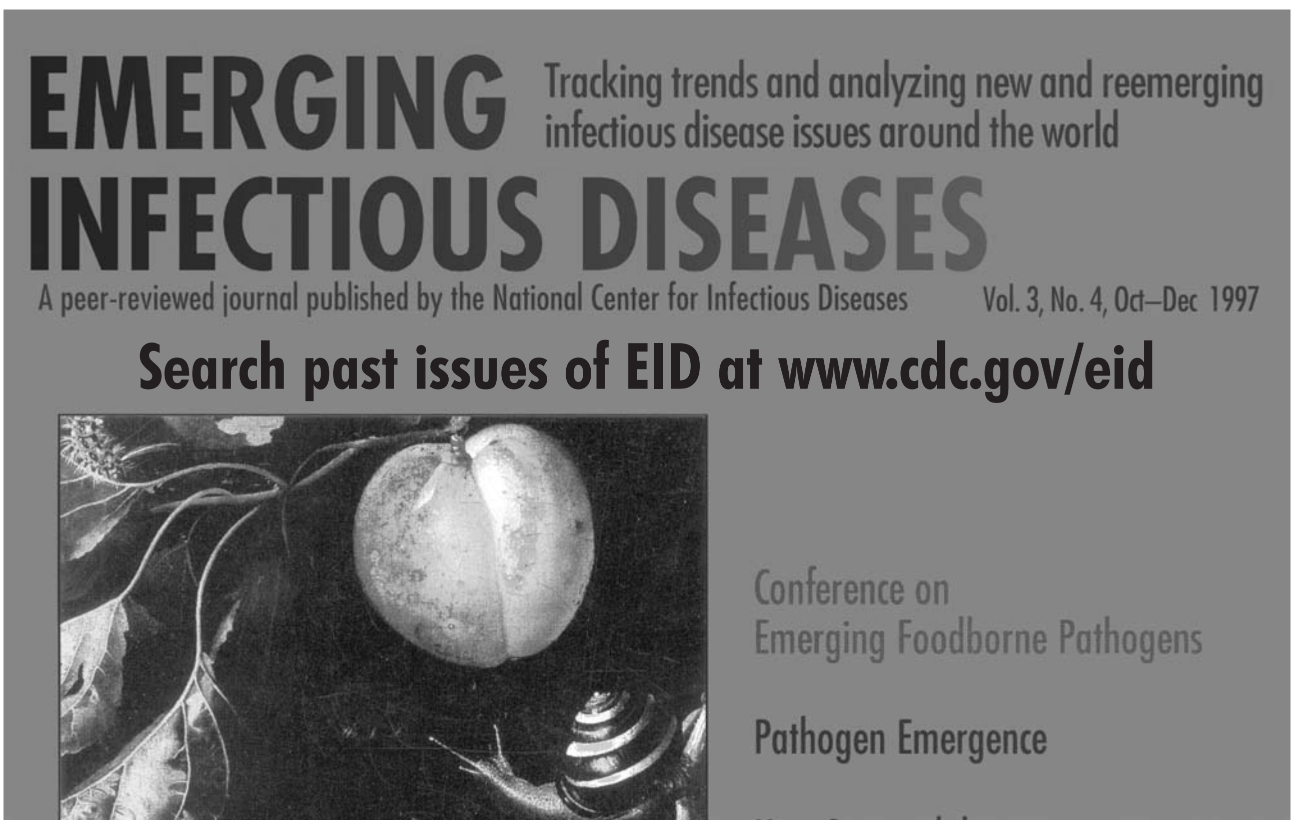

Conference Summary

W135 Meningococcal Disease in Africa[1]

Epidemic meningococcal disease has occurred in Africa for approximately 100 years and has been recognized as a particular problem in sub-Saharan Africa, "the meningitis belt," since 1963. Despite intervention with plain polysaccharide vaccines, thousands of cases and deaths continue to occur. The circumstances that have driven these epidemics of disease remain poorly understood, but a number of factors are likely to be important, including crowded living conditions, population movements, seasonal factors, and the characteristics of the meningococci circulating at a given time. During the latter half of the 20th century, serogroup A meningococci have been responsible for most epidemic disease in Africa; however, as with other regions of the world, cases caused by serogroup B, C, Y, W135, and X meningococci have been occasionally responsible for epidemics. Some epidemic disease outbreaks have been associated with the annual Hajj pilgrimage (e.g., the spread of serogroup A meningococci during the late 1980s and the spread of W135 meningococci from 2000 onwards). Mass vaccination with serogroup A/C plain polysaccharide vaccines has been used to control outbreaks, once they have been identified, in a number of African countries. However, the efficacy of this reactive approach has been questioned, and the recent occurrence of W135 epidemics, combined with a global shortage of the polysaccharide vaccines, creates renewed urgency for a rational and universal preventive program.

This workshop explored the scientific issues behind the design and implementation of a vaccine strategy for the meningitis belt of Africa focusing on the epidemiology of meningococcal isolates. Epidemiologic studies have provided an increasingly detailed knowledge of meningococcal disease in Africa. This knowledge has led to the identification of three distinct clonal complexes responsible for serogroup A disease in Africa (ST-1 complex, ST-4 complex, and ST-5 complex) with successive large-scale epidemics caused by ST-1 and ST-5 complex. Recent epidemiologic findings have shown that serogroup A meningococci belonging to the ST-5 complex (ST-5 and ST-7) were still responsible for most cases and outbreaks of disease in 2000, 2001, and 2002, with the W135 epidemics caused by bacteria belonging to the ST-11 complex. This complex has previously been associated with serogroup C disease. However, while knowledge of the clonal complexes has provided important information on meningococcal disease in Africa, more detailed isolate characterization has shown that important diversity is overlooked by relying solely on sequence type. Despite the availability of a number of meningococcal typing strategies (including pulsed-field gel electrophoresis, multilocus enzyme electrophoresis, and 16s rRNA typing), to date, no portable method is broadly accepted for identifying subvariants below the level of clonal complex. Funding for fundamental research to improve methods of analysis of diversity and dynamics of these populations is an urgent requirement.

Since 2000, serogroup W135 meningococci (ST-11) have been isolated from sporadic cases in Algeria, Cameroon, Chad, Senegal, Niger, and Central African Republic and at the end of a serogroup A outbreak in 2001 and during a large outbreak during 2002 in Burkina Faso. Carriage studies demonstrated a high rate of carriage of W135 in some affected communities in Burkina Faso. Serogroup X has also been widespread in Africa (1970–2000) from studies in Mali, Niger, Burkina Faso, and Ghana. Serogroup X has been primarily found in healthy carriers but also in occasional epidemics.

These studies highlight the importance of supporting enhanced laboratory surveillance throughout the region to monitor the spread of non-serogroup A meningococci. Polymerase chain reaction may increase case ascertainment, but basic microbiologic testing on a large scale is required.

Several studies have been performed on carriage isolates from pilgrims returning from the Hajj. Since 2000 and the introduction of ST-11 complex, W135 meningococci among carried isolates in North Africa (Sudan, Morocco) was documented. By contrast, despite a small increase in cases associated with the Hajj, rates of disease caused by ST-11 W135 meningococci in Europe remained low since 2000, with some evidence that most activity was limited to the Muslim communities.

One study found that the minority (8%) of W135 (case and carrier) isolates are *O*-acetylated (*O*ac+) in the United Kingdom and that the currently available tetravalent polysaccharide vaccine evokes bactericidal activity against both *O*ac+ and *O*ac- W135 and Y isolates. The relevance of *O*-acetylation to vaccine development remains uncertain.

To plan intervention strategies in Africa, changes in the major vaccine antigen (the capsular polysaccharide) present among epidemic disease isolates should be closely monitored. Fundamental research to understand diversity and dynamics of these important bacterial populations is required. The recent epidemic of W135 and substantial numbers of cases caused by other non-A

[1]Report of a workshop held at the International Pathogenic Neisseria conference in Oslo, Norway, September 5, 2002.

serogroups (X and C particularly) provide uncertainty about the future epidemiology of capsule expression during epidemics. Epidemic meningococcal disease in Africa might no longer be thought of as a peculiarity of serogroup A meningococci. The central idea from the workshop was that a comprehensive vaccine (i.e., a multivalent-conjugate) was the optimal approach to controlling epidemic disease in the meningitis belt of Africa. Even this approach may fail, given the remarkable adaptability of this variable organism. Further outbreaks of W135, as well as serogroup A, might occur in the region, and contingency planning for control of W135 outbreaks is required.

A sustainable vaccine program for Africa is needed to prevent future epidemics. Conjugate vaccines provide the possibility of generating protective immunity from infancy and ending epidemic disease. Such vaccines have now been developed by pharmaceutical companies in industrialized nations. However, the challenge is the delivery of effective and affordable vaccines in sub-Saharan Africa, which has not so far been possible in collaboration with major pharmaceutical manufacturers. Practical and economic difficulties exist in delivering an affordable tetravalent ACYW conjugate vaccine for Africa, which, as outlined above, is an important objective. The Meningitis Vaccine Project will support the development of an affordable monovalent serogroup A conjugate polysaccharide vaccine in partnership with a developing country manufacturer. In the long term, this approach allows the possibility of sustainable prevention of epidemics in the region and is of great importance. This workshop concluded that a monovalent serogroup A strategy could leave the population exposed to the risk for further non-A epidemics and that strategies that include other serogroups, particularly W135, need to be put in place as soon as is possible.

Development of an affordable vaccine for Africa cannot be achieved quickly. Discussion of the urgent issue of vaccines for control of epidemics of meningococcal disease in the next few years was not possible during the workshop. The current polysaccharide vaccine shortages raise the possibility that epidemic meningococcal disease continue with no intervention available. ACYW-conjugate vaccines are in development by several major vaccine manufacturers; however, without a market to drive production of millions of doses for sub-Saharan Africa, many more people might die before an affordable vaccine can be delivered by the Meningitis Vaccine Project.

Acknowledgments

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Upcoming Infectious Disease Conferences

November 9–13, 2003
6th OIE Seminar on Biotechnology and 11th International Symposium of the World Association of Veterinary Laboratory Diagnosticians
Office International des Epizooties
Bangkok, Thailand
Contact: 33 (0)1 44 15 18 88
email: oie@oie.int
Web site: www.oie.int

November 15–19, 2003
131st Annual Meeting, American Public Health Association
San Francisco, CA
Contact: APHA Convention Services
(202) 78-05600
Web site: http://www.apha.org

December 2–3, 2003
Course on Current Issues in Clinical Tropical Medicine and Travelers' Health
American Society of Tropical Medicine and Hygiene
Philadelphia, PA
Contact: ASTMH
Phone: 847-480-9592
email: astmh@astmh.org
Web site: http://www.astmh.org

December 3–7, 2003
American Society of Tropical Medicine and Hygiene
52nd Annual Meeting
Philadelphia, PA
Contact: 847-480-9592
email: asthm@astmh.org

December 31, 2003
Sheikh Hamdam Bin Rashid Al Maktoum Award for Medical Sciences
Research Excellence in Emerging Viral and Prion Diseases
Contact: The General Secretariat
Phone: 971-4-227-5888
Fax: 971-4-227-2999
email: shhaward@emirates.net.ae
Web site: http://www.hmaward.org.ae

Francisco José de Goya y Lucientes (1746–1828). Cat Fight (1786–1788)
Model for tapestry intended to decorate the dining room of the Prince of Asturias 1.40 m x 4.38 m
Museo Nacional Del Prado, Madrid, Spain

"Imagination abandoned by reason produces impossible monsters; united with her, she is the mother of the arts," wrote Goya in the subtitle of his etching The Sleep of Reason Produces Monsters. Imagination, its origins and its limits, fascinated Goya and permeated his work, outweighing tradition and ranking him "first of the moderns." Fantasy and invention marked the course of a career during which he revolutionized tapestry design, excelled in portraiture, and along with Dürer, Rembrandt, and Whistler, became one of the greatest graphic artists of all time (1).

Goya was born in the village of Fuendetodos near Zaragoza, in northern Spain, the son of an altar gilder. At 14, he was apprenticed to a local painter and then traveled to Italy, where he learned the decorative manner of rococo. Early in his career, as designer for the royal factory in Madrid, he turned his keen observations of human behavior into innovative tapestry designs depicting scenes of everyday life. He also favored bold new techniques in oil, fresco, and drawing. Influenced by neoclassicism and the works of Velázquez, he moved away from the Baroque toward a realistic portrayal of his time, which he enriched with incisive satire, fantastic visions, and his own interpretation of human nature (2).

Goya was an enigmatic figure. The painter of three generations of kings, he mastered charm and diplomacy, was linked to power, and achieved popular success. Yet, he remained an outsider, often at odds with the court, the monarchy, and the church. His royal portraits were filled with candor and honesty to the point of caricature, his religious subjects were ambiguous and marked by earthy realism, and his women in the flesh caught the attention of the Spanish Inquisition. Many of his original prints, which influenced such masters as Delacroix, Manet, and Picasso, were not seen until after his death because they were deemed too critical of the political and religious order of his day.

Sweeping life changes over Goya's 60-year artistic career are reflected in his work, whose pioneering emotional content influenced the course of 19th and 20th century art. The Spain in which he had been successful without rival in his early years disappeared during the Napoleonic wars. Seven of his children died before reaching adulthood. And serious illness, compounded by his wife's death and other adversities, left him weak, disillusioned, and destitute near the end of his life (3).

Goya's illness, perhaps saturnism caused by toxic fumes from lead salts in the paint he used, brought chronic headaches and permanent hearing loss. Overcome with pessimism, isolated, and traumatized, he created his own aesthetic, turning personal demons into deadly universal themes and painting horrific fantasies laced with caustic social commentary. A collection of 84 prints (Caprices, 1799) satirized the clergy, the nobility, and society's foibles and vices, not the least of them superstition, ignorance, and fear. Another series of 82 realistic etchings (Disasters of War, 1810–1814) chronicled the atrocities following the violence and devastation of the Napoleonic invasion of Spain (1).

Goya was an artist of contradictions and opposites. Along with the bizarre, he painted the comical, along with darkness, bright light. As a result, his images provoke at once fear and delight, sadness and ghoulish mirth. With growing pessimism, he painted bizarre scenes populated with outlandish creatures and monsters engaged in witchcraft, cannibalism, and carnage. Demons, lunatics, lynxes, corpses, and gore allude to human preoccupation with death (4). Goya's imagination, without abandoning reason, harnessed the supernatural into a troubling display of the unconscious and the irrational.

Animals, embodying human weaknesses, social tension, tragedy, the premonition of death, or some other insidious danger, appear repeatedly on a stage of constant hostility and conflict. At their most menacing, they exercise extraordinary powers and influence. In the Cat Fight, on this month's cover of Emerging Infectious Diseases, bellicose cats, frozen in time, apropos of nothing, engage the spectator through pity and terror.

Perched it seems on the edge of the world against an eerie void, these wild creatures stand in electrifying anticipation of trouble. Puffed, curled, glowering, almost translucent against the airless, depthless, oppressive space, they have lost their natural self-directedness. Like marionettes in the hands of an expert puppeteer, they seem to float in fluorescent space, awaiting instructions for the next move in what is certain to be a bloody contest. The brilliant yellow and red highlights reinforce the dreamlike unreality of the scene (4).

Goya's exquisite pessimism, brought on by debilitating illness, guided his exploration of the fundamental mystery of human behavior. Contemporary investigations of the biologic as well as psychological motivators of behavior are guided by research, which now implicates infectious agents in what was long thought "madness." For example, *Toxoplasma gondii,* a single-celled parasite that begins and completes its life cycle in domestic cats, can alter behavior in animals and produce psychotic symptoms in humans (5). *T. gondii*, which causes toxoplasmosis in cats and other mammalian species, may be contributing to some cases of schizophrenia.

Polyxeni Potter

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EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.9, No.12, December, 2003

Upcoming Issue

For a complete list of articles included in the December issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm

Look in the December issue for the following topics:

Emerging Infectious Diseases in Mongolia

Raccoon Roundworm Eggs in Homes and Risk for Larva Migrans Disease

Comparative Molecular and Microbiologic Diagnosis of Bacterial Endocarditis

Emerging Genotype (GGIIb) of Norovirus in Drinking Water

Global Distribution of Rubella Virus Genotypes

Mycobacterium tuberculosis Beijing Genotype, Denmark

Multidrug-resistant *Mycobacterium tuberculosis* in HIV-Infected Persons

Intensity of Rainfall and Severity of Melioidosis, Australia

Scrub Typhus Reemergence in the Maldives

The Rabbit as New Reservoir Host of Enterohemorrhagic *Escherichia coli*

Alexander the Great and West Nile Virus Encephalitis

West Nile Virus in Mexico: Evidence of Widespread Circulation Since July 2002

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's home page at http://www.cdc.gov/eid.

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and e-mail address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables and Figures. Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (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.