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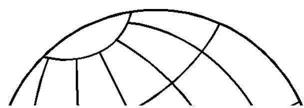
Cover: A mosaic from Louis Pasteur's crypt (1896, Institut Pasteur) representing the well-known episode of the shepherd Jean-Baptiste Jupille struggling against a rabid dog. The shepherd was the second person ever to be vaccinated against rabies. The crypt, modeled on a Byzantine mausoleum, was decorated in a symbolist style by noted French artists of the end of the 19th century. Each mosaic depicts one of Pasteur's main accomplishments (rabies, silkworms, wine, veterinary vaccines). Louis Pasteur rests in the crypt located in the Institut Pasteur in Paris. Printed with permission of the Institut Pasteur.



Bacterial Resistance to Antimicrobial Agents: Selected Problems in France, 1996 to 1998

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Dr. Courvalin is professor at the Institut Pasteur, where he directs the French National Reference Center for Antibiotics and heads the Antibacterial Agents Unit. Dr. Courvalin specializes in the genetics and biochemistry of antibiotic resistance. His research has led to revision of the description of the natural dissemination of antibiotic resistance genes. His laboratory demonstrated that pathogenic bacteria can promiscuously exchange genetic material conferring antibiotic resistance, documented that conjugation could account for dissemination of resistance determinants between phylogenetically remote bacterial genera, elucidated the transposition mechanism of conjugative transposons from gram-positive cocci, and recently, obtained direct gene transfer from bacteria to mammalian cells.

Surveillance of antibiotic resistance in human pathogens has long been performed in France. Existing surveillance relies on national reference centers dedicated to various bacterial genera and on networks of volunteer medical microbiologists, mainly in general hospitals but also in private laboratories. Regional data (often initiated at the request of and funded by the pharmaceutical industry) have been available since the early 1950s. Because of the major health problems caused by antibiotic resistance in the last few years, attempts have been made to organize a national surveillance program similar to those being established in other European countries. Although this update reviews recent data from France, the representativeness of the data has not been assessed. In addition, these are raw data, and their clinical importance remains to be seen; for example, the contribution of bacterial isolates to infection or colonization is, in most instances, unknown.

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Evolution of Antibiotic Resistance in Hospitals

Dissemination of β-Lactamases in Gram-Negative Bacilli

In France, β-lactamases and fluoroquinolones are the most frequently prescribed antibiotics in *Enterobacteriaceae* infections. A multicenter study (14 hospitals) across the country analyzed the antibiotic susceptibility of 2,507 and 2,312 consecutive, nonrepetitive enterobacteria responsible for infection in 1996 and 1997, respectively (1,2). Strains were isolated from inpatients (86%) in intensive care (ICU) (12%), surgical (17%), medical (37%), and geriatric (9%) units. The majority of isolates were from urine (71%), pus (9%), and bronchopulmonary specimens (8%). *Escherichia coli* (64%) was isolated most frequently, mainly in outpatients, whereas *Klebsiella* spp. and *Enterobacteriaceae* with inducible β-lactamases predominated in ICUs. Resistance of *E. coli* to amoxicillin and cefotaxime was 47% and 0.5%, respectively. In 1997, the frequency of isolates producing an extended-spectrum β-lactamase varied by species: in *Enterobacter aerogenes,* 56%; in *Klebsiella pneumoniae,* 15%; and in *E. coli,* 0.5%. The incidence differed within and between hospitals. Such strains arise in response to the selective pressure exerted by use of extended-spectrum cephalosporins (3); infections with

such strains have also been associated with hospitalization in ICUs. Production of β-lactamases resistant to β-lactam-enzyme inhibitor combinations in *E. coli* was approximately 3.5% (2). Susceptibility to fluoroquinolones was high (66%-97% ciprofloxacin-susceptible) except in *E. aerogenes* and *Serratia marcescens* (35%-52% ciprofloxacin-susceptible) (1).

The organisms most frequently isolated in ICUs in 1995 belonged to the family *Enterobacteriaceae* (59%) and *Pseudomonas aeruginosa* (25%) (4). In 1997, 1,362 nonrepetitive *P. aeruginosa* (5% of all clinical isolates) were collected in 13 teaching hospitals (5). The lowest rates of susceptibility to ceftazidime (<75%), amikacin (70%), and ciprofloxacin (65%) were observed with serotype 12 (the fourth main serotype). Among penicillinase-producing strains, the percentages of resistance to amikacin and ciprofloxacin were 80% and 93%, respectively; these figures were substantially higher in β-lactam-resistant *P. aeruginosa* than in susceptible strains.

Spread of Methicillin-Resistant and Gentamicin-Susceptible *Staphylococcus aureus*

Methicillin-resistant *S. aureus* (MRSA) is one of the most frequent nosocomial pathogens in France as in the rest of the world. Surveys conducted in hospitals in Paris and surroundings found that MRSA decreased from 42% in 1992 to 37% in 1997 and that the incidence of MRSA colonization-infection (approximately 0.65 per 100 admissions) also decreased after national recommendations against dissemination of multidrug-resistant bacteria were implemented (6,7). However, a survey of 26 geographically representative hospitals found that the incidence of gentamicin-susceptible MRSA progressively increased because of the presence of a predominant clone (H. Lelièvre, G. Lina, M.E. Jones, et al., unpub. obs.). The epidemiologic situation in France is complex. The endemic aminoglycoside-resistant MRSA strain persisted while new clones became endemic in hospitals, perhaps after changes in the use of aminoglycosides (decrease of gentamicin and increase of amikacin consumption) (8). The first vancomycin-intermediate *S. aureus* was isolated in a French hospital in 1995; no other cases of MRSA with reduced susceptibility to vancomycin have been reported (9).

Most Frequent Macrolide-Resistance Mechanisms among Staphylococci

Over 3 weeks in 1995, 607 staphylococci were collected in 32 hospitals (10). Of these, 45.5% of the *S. aureus* and 54% of the coagulase-negative staphylococci were resistant to methicillin, and 71.5% of MRSA were resistant to macrolides. Of these MRSA strains, 75% were constitutively resistant, whereas 76% of MSSA were inducibly resistant. A similar distribution (61% vs. 27.5%) was observed among coagulase-negative staphylococci. Resistance to at least one of the macrolide, lincosamide, and streptogramin antibiotics (88%) was due to the presence of the *ermA* and *ermC* genes, which confer resistance by modifying the ribosomal target. The *ermA* gene was more common in MRSA (57.6%) than in MSSA (5.6%), where *ermC* was predominant (20.1%). *ermC* was also common among methicillin-susceptible coagulase-negative staphylococci (14%). Only a few strains had the *ermB* gene, which is found in animal strains. Macrolide resistance by efflux due to acquisition of the *msrA* gene was more prevalent in coagulase-negative staphylococci (14.6%) than in *S. aureus* (2.1%). The incidence of lincomycin-resistant but macrolide- and streptogramin-susceptible staphylococci was low: 0.2% in *S. aureus* and 4.6% in *Staphylococcus epidermidis* (11). The prevalence of pristinamycin-resistant (and also most probably quinupristin/dalfopristin-resistant) strains remained low because of the low incidence of resistance to streptogramins type A (pristinamycin has limited use in France).

Dissemination of Resistance in the Community

Effect of Antibiotics on Oropharyngeal Flora

Antibiotic Resistance in *Streptococcus pneumoniae*

The National Reference Center for Pneumococci determined the susceptibility to antibiotics of 2,837 *S. pneumoniae* isolated in 1997. The incidence of *S. pneumoniae* with reduced susceptibility to penicillin G increased from 3.8% in 1987 to 48% in 1997 (12). Whereas 53% of all strains were resistant to macrolides, 80% of penicillin-resistant strains were macrolide-resistant; 15% of all strains (versus 51% of penicillin-resistant strains) were resistant to tetracycline, and 10% (versus 66%), respectively,

were resistant to trimethoprim-sulfamethoxazole (F. Goldstein et al., unpub. data). According to a 1997 survey of 18 regional laboratories in France (11,757 strains collected), 27% had intermediate levels of resistance to penicillin G, and 13.5% were fully resistant. The rates varied considerably by region (highest in southwest and central France), age (highest [37.4% penicillin G-intermediate and 21.5% resistant] in children <16 years old), and specimen source (highest in middle ear and sinus specimens) (13).

Resistance to Macrolides in β-Hemolytic Streptococci

In 1995, a national survey in 98 hospitals of invasive infections due to *Streptococcus pyogenes* found that 5.2% to 9.8% of the strains isolated from blood were erythromycin-resistant (A. Bouvet, pers. comm.) (14).

Vaccination against and Resistance in *Haemophilus influenzae* Type b

To monitor the trends in *H. influenzae* meningitidis and the prevalence of resistance, the National Reference Center conducted a survey of approximately 80 hospitals (15). Since vaccination for Hib invasive infections began in 1993, the percentage of capsulated isolates has decreased 5% per year. Moreover, resistance to antimicrobial drugs decreased among Hib and increased among noncapsulated strains isolated from upper and lower respiratory tract infections. The percentage of β-lactamase-producing *H. influenzae* increased progressively from 22% in 1992 to 35% in 1997, with a similar evolution for kanamycin resistance. Tetracycline and chloramphenicol resistance remained stable in 1997—less than 10% and 2%, respectively (15,16).

Antibiotic Resistance in *Neisseria meningitidis*

Meningococcal resistance to antibiotics is emerging in France. The incidence of *N. meningitidis* with reduced susceptibility to penicillin G (MICs from 0.125 mg/L to 1 mg/L) increased from less than 1% in 1991 to 18% in 1996 (17,18). The strains belonged to various serogroups; most belonged to serogroup B, none produced a β-lactamase, and all were susceptible to cefotaxime and ceftriaxone. Resistance to rifampin, used for prophylaxis of secondary cases in France, remained low (0.02% in 1996).

Effect of Antibiotics on Digestive Flora

Antimicrobial Resistance in *Helicobacter pylori*

Susceptibility testing of *H. pylori* from 535 patients with a positive CLO test was performed in 1997 (19). Depending on the method, the percentages of clarithromycin resistance (disk-agar diffusion or MIC determination by agar dilution) and metronidazole resistance (breakpoint method at 8 mg/L or MIC determination) varied from 14.3% (95% confidence interval [CI] 11.5-17.6) to 14.0% (95% CI 11.2-17.3) and from 30.5% (95% CI 25.6-34.5) to 23.6% (95% CI 20.1-27.5), for the two antibiotics, respectively. No resistance to amoxicillin was observed.

Fluoroquinolone Resistance in *Campylobacter* and *Salmonella* Hadar

The evolution of antimicrobial resistance in *Campylobacter jejuni* and *C. coli* is worrisome. Between 1986 and 1997, 2,713 strains of *C. jejuni* (68% of total Campylobacter isolates) were isolated from stool (94%) and blood (4%) and studied (20). Between 1993 and 1997, fluoroquinolone resistance increased from 7.4% to 32% in *C. jejuni* and from 11.8% to 52% in *C. coli.* The high resistance rate to quinolones makes them ineffective in therapy of *Campylobacter* infections. These resistance rates are similar to those in other countries (e.g., Spain, the United Kingdom) (21,22). However, the prevalence of macrolide-resistant strains remains low (3.6%). The high incidence of multidrug-resistant *Salmonella* Typhimurium DT104 (12 atypical), with 82% resistance to ampicillin, streptomycin, sulphonamide, tetracycline, and chloramphenicol, is the most serious epidemiologic problem of the last decade in France (23). The incidence of *Salmonella* Hadar is increasing, and the percentages of amoxicillin- and fluoroquinolone-resistant strains in 1997 were 72% and 75%, respectively. Fluoroquinolone resistance had not been observed before 1987 in France, Spain, and the United Kingdom. This was before concomitant introduction of ciprofloxacin into clinical use and enrofloxacin into veterinary use (in particular in the poultry industry) in the late 1980s. More than 50% of *C. jejuni* and *S.* Hadar, the most frequent serotype associated with poultry, are now fluoroquinolone-resistant in these countries.

The situation is different in Sweden, where fluoroquinolones are not readily available. Therefore, guidelines for the prudent use of antibiotics (in prophylaxis or therapy) should be developed that respect the indigenous flora of humans and animals.

New Types of Resistance in Enterococci

The increase in the incidence of glycopeptide-resistant enterococci (GRE) isolated from hospitalized patients throughout the United States has not been observed in France. A multicenter study in 1993 showed a very low incidence of GRE: 0.2% among 251 enterococcal clinical isolates and 7.5% among *Enterococcus faecium* (24). Study of 24 ICUs in 1994 determined that the prevalence of GRE colonization in patients' fecal flora was approximately 2%, 30% of which had been present at admission. No nosocomial infection due to GRE was observed (25). GRE have been identified in human food of animal origin (40% of GRE were isolated from uncooked meat) in a French study conducted in military cafeterias in 1997 (26). Thus, food may represent a major source of human colonization with GRE in France. GRE strains isolated in France were also resistant to ampicillin, tetracycline, and macrolides. However, the percentage of high resistance levels to gentamicin among GRE was comparable to that among glycopeptide-susceptible enterococci.

Antibiotic Resistance in *Bacteroides fragilis*

Studies of antibiotic resistance in anaerobic pathogens indicate stability of resistance to carbapenems (imipenem) and nitroimidazole antibiotics (27,28). In 1998, fewer than 2% of all *B. fragilis* from 39 hospitals were resistant to metronidazole (MICs >8 mg/L), and the number of imipenem-resistant strains remained low. However, this gene reservoir requires surveillance of resistance in *B. fragilis* infections because of the use of these antibiotics in therapy.

Other Bacteria

Antibiotic Resistance in *Neisseria gonorrhoeae*

The number of *N. gonorrhoeae* strains identified by the National Reference Centre for Sexually Transmitted Diseases fell sharply from 1986 to 1990 (by 81%) and more slowly from 1990 until 1999 (by 55%) (29). The number of anorectal gonococcal infections reached a plateau from 1995 to 1997 but increased again in 1998, mostly in the Paris/Ile-de-France region (V. Goulet, P. Sednaoui, et al., unpub. data). An increasing percentage of *N. gonorrhoeae* displayed diminished sensitivity to penicillin G and to tetracycline. In 1997, 15% and 30% of *N. gonorrhoeae* were resistant to penicillin G (MIC $\geq$ 2 mg/L) and tetracycline (MIC 2 $\geq$ mg/L and < 16 mg/L), respectively, by chromosomal mutation. In contrast, the percentage of strains with plasmid-mediated resistance to penicillins and tetracycline has remained stable at approximately 15% since 1994. No ceftriaxone, spectinomycin, or ciprofloxacin resistance was found until 1997, when the first ciprofloxacin-resistant strains (MIC=1 mg/L) were isolated.

Conclusions

Antibiotic resistance trends in France are for the most part similar to trends in other European countries but with some peculiarities. For instance, fluoroquinolone resistance in *Salmonella* spp. and *Campylobacter* spp. is a problem throughout Europe. However, methicillin resistance in *Staphylococcus* is more common in France than in the Scandinavian countries, although it has started to decrease because of reinforcement of hygiene measures since 1992. Also, heavy use of 16-membered macrolides has selected for resistance in gram-positive cocci by ribosomal modification rather than by efflux. In pneumococci, decreased susceptibility to penicillins is as common in France as in Spain, but the incidence of resistance to macrolides is the highest in Europe. The public health problems caused in France by bacterial resistance to antibiotics are clearly distinct from those in North America. The incidence of enterobacteria producing extended-spectrum β-lactamases and glycopeptide-resistant enterococci remains rather low in France, as in most other European countries. In the United States, the high incidence of nosocomial GRE infections is probably caused by the heavy nosocomial use of vancomycin, particularly in hematology wards and for the prevention of colitis due to *Clostridium difficile*. In contrast, no intestinal carriage of such strains is found in the general population. The situation in Europe mirrors that in the United States. In Europe, the prevalence

of nosocomial GRE infections remains low, but colonization of the population is substantial, possibly because of the use of a vancomycinlike antibiotic (avoparcin) as an animal food additive. This example stresses the need for a multidisciplinary approach to surveillance of bacterial resistance to antibiotics.

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The Cost Effectiveness of Vaccinating against Lyme Disease

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To determine the cost effectiveness of vaccinating against Lyme disease, we used a decision tree to examine the impact on society of six key components. The main measure of outcome was the cost per case averted. Assuming a 0.80 probability of diagnosing and treating early Lyme disease, a 0.005 probability of contracting Lyme disease, and a vaccination cost of $50 per year, the mean cost of vaccination per case averted was $4,466. When we increased the probability of contracting Lyme disease to 0.03 and the cost of vaccination to $100 per year, the mean net savings per case averted was $3,377. Since few communities have average annual incidences of Lyme disease >0.005, economic benefits will be greatest when vaccination is used on the basis of individual risk, specifically, in persons whose probability of contracting Lyme disease is $\geq$0.01.

Lyme disease, caused by infection with *Borrelia burgdorferi*, is the most common tick-borne disease in the United States and Europe (1-3). In the United States, the disease has spread slowly, and the number of cases in disease-endemic areas has increased (4-6). Most Lyme disease patients become infected with *B. burgdorferi* near their homes, while engaged in property maintenance, recreation, and relaxation (7). Occupational and recreational activities away from home may also pose a risk (8). Lyme disease prevention based primarily on avoidance of tick bites, use of repellants, early detection and removal of attached ticks, and tick control has not substantially reduced disease incidence (4-6). Therefore, preventive vaccines have been of considerable interest. Results of randomized and blinded phase-III field trials with recombinant *B. burgdorferi* outer surface protein A (rOspA) vaccines indicate that they are safe and efficacious (9,10). On December 21, 1998, the U.S. Food and Drug Administration licensed one of the vaccines (LYMErix, SmithKline Beecham Biologicals, Reixensart, Belgium) for use in the United States (11).

We present the results of an analytic model that evaluates the cost effectiveness of using a vaccine to protect against Lyme disease in the United States.

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The Model

Using a computer-based spreadsheet (Excel 5.0 for Windows, Microsoft), we constructed a decision tree (12) to evaluate the cost per case averted (cost effectiveness) to society of vaccinating against Lyme disease (Figure 1). Many data needed to determine the cost effectiveness of vaccinating against Lyme disease are unvalidated, unavailable, or available only from very small databases. Thus, rather than calculate a single estimate of cost per case averted, we examined the effect of combinations of six inputs: cost of vaccination; annual probability of contracting Lyme disease; costs of successfully treating either early symptoms of Lyme disease or one of three sequelae (cardiovascular, neurologic, arthritic); probability of diagnosing and treating early symptoms; probability of sequelae due to early infection; probability of sequelae due to late, disseminated infection.

Mathematically, we examined the effect of altering the values of the inputs by using specialized computer software (@Risk, Palisade Corp., Newfield, NY) (13) that employs Monte Carlo methods (14-16). To use these methods, the researcher defines probability distributions for selected inputs by using available data and

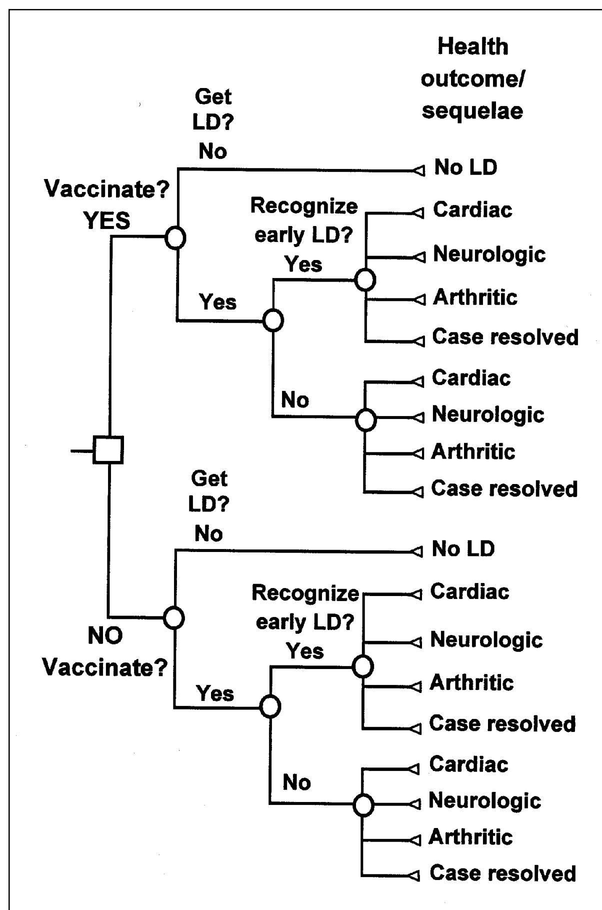

Figure 1: Decision tree to model the cost effectiveness of vaccinating a person against Lyme disease.

enters them into the computer program. For each iteration of the program, an algorithm selects input values from the probability distributions, calculates the net result (here, the cost per case averted), and stores that result. After all iterations (typically 1,000 to 5,000) are completed, the program produces a probability-based distribution of the net result, which can then be used to report statistics such as mean, median, and 5th and 95th percentiles.

Cost Effectiveness Formula

The formula used to calculate the cost per case of Lyme disease averted was as follows:

$$\text{Cost per case averted} = \frac{\text{\$ of vacc+\$of LD with vacc-\$ of LDw/o vacc}}{\text{Prob LD w/o vacc-Prob LD with vacc}}$$

where $ = cost; vacc. = vaccination; LD = Lyme disease; and prob. = probability. The numerator is the cost of vaccination less any savings resulting from the reduced probability of contracting the disease (decreased incidence) due to vaccination. If the vaccine is not 100% effective in preventing Lyme disease (i.e., if the term Prob. LD with vacc. >0), treatment costs may still be incurred after vaccination. The cost of a case of Lyme disease is the weighted average cost of all health outcomes (Figure 1), where the weights are the probabilities of those outcomes (12). The denominator reflects the change in the probability of Lyme disease due to vaccination.

Vaccine Timeline

Although experiments have shown that a Lyme disease vaccine using rOspA is safe and immunogenic in both animals and humans (17-23), no data have been published concerning the decrease in antibody levels over more than 20 months (9). Phase-III vaccine field trials used a 0-, 1-, and 12-month immunization schedule, and antibody levels dropped almost 10-fold between the month after the second dose and just before the third dose at month 12 (9). The third dose at month 12 boosted antibodies to levels higher than measured at month 2, but these declined by half by month 20 (9). We assumed, therefore, that an annual booster dose would be required and that the cost-effectiveness model would be repeated annually. When calculating annual benefits, however, we included the discounted savings of preventing Lyme disease that may generate multiyear sequelae.

Lyme Disease Symptoms and Sequelae

The most common symptoms of infection with *B. burgdorferi* can be categorized as early localized disease (stage I); early disseminated disease (stage II); and later-stage sequelae of disseminated infection (stage III) (24). Stages I and II correspond to the branches labeled "Recognize early LD? Yes" in Figure 1, and stage III corresponds to the branches labeled "Recognize early LD? No." Most early symptoms of Lyme disease respond promptly and completely to short courses of oral antibiotics (25-27). Later-stage sequelae, however, may require costly, more prolonged treatment, sometimes repeated courses of treatment using intravenous cephalosporins, and may not be completely eliminated (28).

If a person, vaccinated or unvaccinated, contracts Lyme disease, the model allows for one of four possible categories of outcomes (Figure 1)

(29-31): cardiovascular sequelae (e.g., high-grade atrioventricular blocks); neurologic sequelae (e.g., isolated cranial nerve palsy, meningitis); arthritic or rheumatologic/musculoskeletal sequelae (e.g., episodic oligoarticular arthritis, arthralgia); and case resolved (after a course of an oral antibiotic such as doxycycline) with no further complications.

The disseminated stages of Lyme disease may be manifested weeks to months after infection (24). However, few data concerning the duration of such sequelae are available. One study, for example, involving 38 patients showed that their long-term clinical sequelae lasted a mean of 6.2 years from onset of disease (32). The use of health-care resources, however, by those patients during that time was not reported. We assumed that cardiovascular sequelae would be treated and resolved in an average of 1 year and that late neurologic and arthritic sequelae would both take an average of 11 years to diagnose and satisfactorily treat to full resolution (initial year of diagnosis and treatment plus 10 years of additional treatment). These assignments of average time are arbitrary and longer than any published average, which maximizes estimated economic benefits of using a vaccine.

Probabilities

We selected three probabilities (0.005, 0.01, and 0.03) of contracting Lyme disease (Table 1) on the basis of data concerning disease incidence in Lyme disease-endemic areas (33-36); the probability of 0.03 is among the highest reported. (Before the risk for Lyme disease was widely recognized, a one-time annual incidence of 10% was reported in a community of 190 people living next to an open nature preserve [37].) Vaccine efficacy in preventing Lyme disease was 50% (95% confidence intervals [CI]: 14% to 71%) after the first two doses and 78% (95% CI: 59% to 88%) after three doses (9,11). We assumed Lyme disease vaccine to be 85% effective, which is near the upper end of the 95% confidence limits and thus maximizes estimated economic benefits. We selected 0.6 to 0.9 as the range of probability of early diagnosis and treatment on the basis of a study on the economic cost of Lyme disease, which included data from an expert panel (38). For the Monte Carlo simulations (14-16), we constructed the distributions describing the probabilities of having one of the three sequelae (due to either early or late disseminated disease) using data from the previously mentioned expert panel (Table 1) (38). The distributions describing cardiac and neurologic complications associated with early Lyme disease are uniform, defined by using minimum and maximum values (39) and reflecting the uncertainty regarding a most likely value (38). All other distributions are triangular (39), with minimum, most likely, and maximum values (Table 1).

Table 1. Probabilities and their statistical distributions

Item	Values	Type of distribution[a]
Probability of contracting LD[b]	0.005, 0.01, 0.03	Fixed intervals[c]
Effectiveness of vaccine	0.85	Fixed
Probability of early detection of LD	0.6 - 0.9	Fixed intervals[cd]
Probability of sequelae[e] if detect LD early		
Cardiac	0 - 0.01	Uniform[f]
Neurologic	0 - 0.02	Uniform[f]
Arthritic	0.02-0.05-0.07	Triangular[g]
Case resolved	Residual[h]	N/A
Probability of sequelae if do not detect LD early		
Cardiac	0.02-0.03-0.06	Triangular
Neurologic	0.02-0.15-0.17	Triangular
Arthritic	0.5-0.6-0.62	Triangular
Case resolved	Residual[h]	N/A

[a]Statistical distribution used in Monte Carlo simulations (14-16).
[b]LD = Lyme disease.
[c]Iterations are run by using different combinations of the probabilities of infection and cost of treatment (Table 2).
[d]The interval between the minimum and the maximum is divided into 0.1 increments.
[e]See text for description of sequelae.
[f]Uniform distribution implies that there is an equal chance that any number between, and including, the minimum and maximum will be used for a given iteration.
[g]Triangular distribution is defined by points of minimum, most likely, and maximum.
[h]The probability of an LD case being successfully resolved (i.e., no further sequelae) is 1 - (sum of the probabilities of cardiac + neurologic + arthritic symptoms).

Vaccination Costs

Although a Lyme disease vaccine has been licensed (11), data are not available on the actual cost of vaccination, which includes costs of the vaccine, its administration, time spent in receiving the vaccine, travel, and treatment of adverse side-effects of vaccination. To allow for variation caused by variables such as location of provider, type of provider, and type of third-party payer, we estimated cost effectiveness by using three costs: $50 per person per year, $100 per person per year, and $200 per person per year.

Few data are available on the costs of treating a case of Lyme disease; only one study (29) has documented the charges in 1989 dollars associated with some sequelae. To adjust charges reported in that study to 1996 prices, we multiplied the charges by a factor of 1.528 (medical care component of the consumer price index) (40). These 1996 prices, however, reflected health-care charges paid by health insurance companies and not necessarily actual economic costs (41,42). Thus, to reflect economic costs, the adjusted prices were multiplied by cost-to-charge factor (the weighted average of the urban and rural hospital cost-to-charge ratios used by the U.S. Federal Health Care Finance Administration [43]) of 0.53. Data describing indirect costs, particularly lost productivity, associated with sequelae were unavailable. We therefore assumed that Lyme disease–related cardiac sequelae would cause 14 days of lost productivity, and neurologic and arthritic sequelae would each cause 21 days of lost productivity per year. Each day of lost productivity was valued at $100 (the average income of a workday [1990 dollars inflated to 1996 values] weighted by the age and sex composition of the U.S. workforce) (44). Because we assumed that late-stage neurologic and arthritic complications may take up to 11 years to completely resolve, the 1-year cost estimates for treating these sequelae were replicated over 11 years and then discounted at 3% to the base year (Table 2).

We also altered the estimate of Magid et al. (29) of charges for resolving a case of Lyme disease without complications by doubling the number of office visits to two ($25 each visit) and allowing for 5 hours of lost productivity ($62) for a total of $161 (Table 2). In comparison, a recent study concerning Lyme disease on the eastern shore of Maryland found the median charge for the diagnosis and treatment of Lyme disease was $199 (45); this figure represents charges, and actual economic costs are likely lower than this amount (41,42).

Table 2: Costs of treating one case of Lyme disease and the sequelae due to early and late disseminated disease

Item	Cost/ year ($)	Length of treat- ment	Total costs[a] ($)
Case resolved: no sequelae			
Antibiotics	14		
Office visits (2)	50		
Laboratory tests	35		
5 hrs lost work time	62		
Total	161	2-3 wks	161
Sequelae[b] due to early and late disseminated disease			
Cardiac-direct[c]	5,445		
Cardiac-indirect[d]	1,400		
Cardiac-total	6,845	≤ 1 yr	6,845
Neurologic-direct[c]	4,865		
Neurologic-indirect[d]	2,100		
Neurologic-total	6,965	11 yrs	61,243
Arthritic-direct[c]	1,804		
Arthritic-indirect[d]	2,100		
Arthritic-total	3,904	11 yrs	34,354

[a]All costs that occur over more than 1 year are discounted at a rate of 3% per year.
[b]See text for description of the sequelae.
[c]Direct costs are for all medical costs and are derived from the 1-year charges reported by Magid et al. (29), inflated to 1996 dollars (factor of 1.528) (40), and then adjusted by a cost-to-charge ratio of 0.53 (43) (see text for details).
[d]Indirect costs are the valuation of lost productivity due to Lyme disease-related illness, with each day lost valued at $100. For cardiac-related sequelae, it was assumed that 14 workdays were lost, and for neurologic and arthritic-related sequelae, it was assumed that 21 workdays were lost each year.

Sensitivity Analyses

To allow for uncertainty caused by lack of data, we conducted multivariate sensitivity analyses in which we simultaneously altered the assumed effectiveness of the vaccine and the cost of treating sequelae. We altered vaccine effectiveness to either 0.75 or 0.95 (compared with 0.85 in the base case [Table 1]), and we multiplied the total costs for treating sequelae (Table 2) by 0.5 or 1.5. For example, for neurologic sequelae, the latter multiplier is equivalent to increasing the days of lost productivity (indirect costs) from 21 days per year to 31.5 days per year. We set the probability of identifying and successfully treating early Lyme disease at 0.80 and the cost of vaccination

at $100 per year. The estimates generated by these sensitivity analyses were compared with those generated using the base costs, with an assumed vaccine effectiveness of 0.85, and with the same assumptions for probability of identifying and treating early Lyme disease and cost of vaccination as used in the sensitivity analyses.

Findings

Assuming a 0.005 probability of contracting Lyme disease, a 0.80 probability of diagnosing and treating early Lyme disease, and a $50 per year cost of vaccination, the mean cost per case averted was $4,466 (5th percentile = $5,408; 95th percentile = $3,587) (Figure 2). The 5th and 95th percentiles were calculated as part of the Monte Carlo simulations (14-16). To enhance clarity, the 5th and 95th percentiles were not plotted on Figure 2. Increasing the cost of vaccination to $100 per year increased the mean cost per case averted to $16,231 (5th = $17,267; 95th = $15,298) (Figure 2). At a cost of vaccination of $200 per year, the mean cost per case averted was $39,761 (5th = $40,858; 95th = $38,830) (Figure 2).

With a 0.01 probability of contracting Lyme disease and a 0.80 probability of correct diagnosis and treatment of early disease, the mean savings per case averted was $1,416 when the cost of vaccination was $50 per year. Vaccination resulted in a net cost of $4,467 when the cost of vaccination was $100 per year and a net cost of $16,231 when the cost of vaccination was set at $200 per year (Figure 2).

When we set the probability of contracting Lyme disease at 0.03 and used the same probability of diagnosis as before (0.80), the mean savings per case averted was $5,337 when the cost of vaccination was $50 per year and $3,377 when the cost of vaccination was $100 per year. The net cost per case averted was $545 when the cost of vaccination was $200 per year (Figure 2).

When the costs of treating sequelae were reduced by half of base costs, at a 0.01 probability of contracting Lyme disease, the average cost of averting one case was $9,684 when vaccine effectiveness was assumed to be 0.75 and $6,877 when vaccine effectiveness was assumed to be 0.95 (Table 3). These are 117% and 54% higher, respectively, than the costs calculated using the base costs (Table 3).

When the costs of treating sequelae were increased to 1.5 times base costs, the equivalent

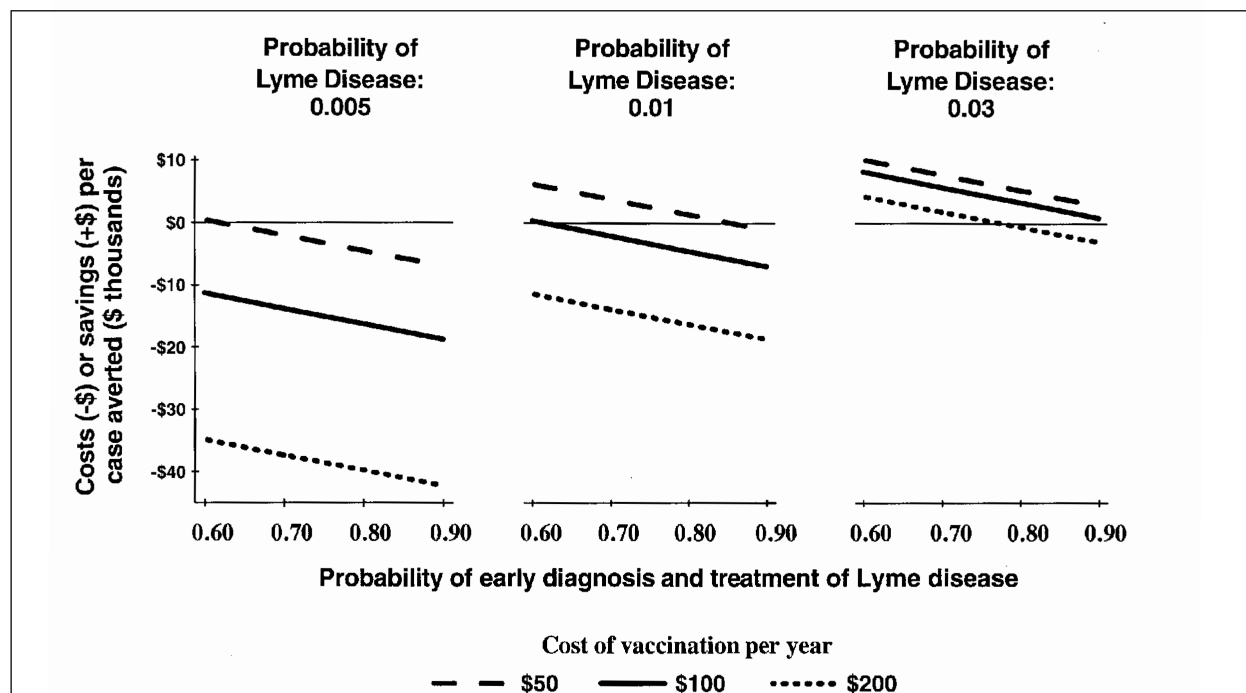

Figure 2: Average cost effectiveness of vaccinating a person against Lyme disease with changes in the cost of vaccination, probabilities of identifying and treating early Lyme disease, and probabilities of contracting Lyme disease. A negative value indicates that vaccinating a person will result in a net cost to society, while a positive value indicates a net savings to society. The results shown are the means from Monte Carlo simulations (see Table 1 and text for further details). Vaccine assumed 85% effective (Table 1).

Table 3. Sensitivity analyses: Cost or savings per case averted (5th, 95th percentiles) by altering assumed vaccine effectiveness and the cost of treating Lyme disease sequelae[a]

Probability of Lyme disease	Base treatment costs[b] x 0.5		Base treatment costs[c]	Base treatment costs[b] x 1.5	
	Vaccine effectiveness[d]			Vaccine effectiveness[d]	
	0.75	0.95	0.85	0.75	0.95
0.005	23,018 (23,527; 22,556)	17,404 (17,947; 16,927)	16,231 (17,283; 15,261)	15,720 (17,249;14,286)	10,105 (11,641;8,703)
0.01	9,684 (10,178; 9197)	6,877 (7,372; 6,412)	4,467 (5,531; 3,487)	2,386 (3,846; 958)	Net savings[e] (1,220; save[e])
0.03	795 (1,303; 330)	Net savings[e] (385; save[e])	Net savings[e] (save; save[e])	Net savings[e] (save;save[e])	Net savings[e] (save; save[e])

[a]These results were generated by setting the probability of detecting and successfully treating early Lyme disease at 0.80 and the cost of vaccination at $100 per year.
[b]Base treatment costs are given in Table 2. The data presented in this table were generated by multiplying the costs in Table 2 by either 0.5 (i.e., reducing costs by half) or by 1.5 (i.e., increasing costs by half).
[c]For comparison, the results using the base costs (Table 2) are presented here, assuming a vaccine effectiveness of 0.85. Figure 2 presents the complete set of results using the base costs.
[d]The initial assumed level of vaccine effectiveness was 0.85 (Figure 2).
[e]Net savings are generated when a person is vaccinated against Lyme disease and the costs saved by not having to treat a case of Lyme disease are higher than the costs of vaccination plus the costs of having to treat a case of Lyme disease that occurs after vaccination. The net savings range from $140 (probability of Lyme disease = 0.03, vaccine effectiveness = 0.95, cost of treating Lyme disease sequelae = 0.5 x base costs) to $7,438 (probability of Lyme disease = 0.03, vaccine effectiveness = 0.95, cost of treating Lyme disease sequelae =1.5 x base costs). Note also that in some instances where mean net savings are calculated, the 5th percentiles are net costs.

cost per case averted was $2,386 at a vaccine effectiveness of 0.75, while a vaccine effectiveness of 0.95 was estimated to generate cost savings (Table 3). The former estimate represents a 47% decrease in cost per case averted compared with the base case (Table 3).

These results show that, as the weighted average cost of treating a case of Lyme disease decreases (increases), the cost per case averted through vaccination increases (decreases). An inspection of the formula to calculate the cost per case of Lyme disease averted, presented in the Model section, shows that, as the term $ of LD w/out vacc (in the numerator) decreases, the cost per case averted must increase.

Conclusions

Because of either lack of data or wide variability in some key variables (e.g., cost of vaccination, risk for Lyme disease), a single answer regarding the cost effectiveness of vaccinating a person against Lyme disease cannot be calculated. The methods we used allow physicians, health-care decision makers, and public health authorities to use Figure 2 and Table 3 to determine the cost effectiveness of vaccination for their specific situations. This simple model can be rerun to provide estimates per case averted for situations not covered in the results presented (e.g., lower or higher probabilities of Lyme disease). The estimates do not include any valuation of a person's willingness to pay for the vaccination.

Relative Importance of Input Variables

The probability of contracting Lyme disease is the most important factor in determining the economic benefit of vaccinating against Lyme disease (Figure 2). The results from Figure 2 and from the sensitivity analyses concerning the costs of treating sequelae and vaccine effectiveness (Table 3) indicate that the next most important variables are the cost of treating sequelae and the probability of early detection and treatment of Lyme disease.

Research Priorities

Given the importance of treatment costs in assessing the cost effectiveness of Lyme disease vaccine, accurate data regarding the cost of treating sequelae should receive high priority when setting a research agenda for Lyme disease. Data concerning the duration of the various forms of long-term sequelae and the indirect costs borne by patients are also important. For both items, research should not focus on obtaining a mean value but rather on collecting sufficient data to describe the probability distribution of these input variables, which could either replace the assumed distributions (Table 1) or be added to the model to further refine the results.

Implications for Public Health Policy

Very few communities have an annual incidence of Lyme disease of 0.005 or higher. From 1992 to 1996, approximately 47% (1,483) of U.S. counties reported at least one case of Lyme disease. However, 148 counties (almost all in the northeastern and northcentral United States [CDC, unpub. data; 1]) reported 90.3% of cases. Connecticut and Rhode Island had the highest cumulative annual incidences of reported Lyme disease, equivalent to probabilities of contracting Lyme disease of 0.000949 and 0.000539, respectively (1996 data) (46). Two studies (47,48) have shown that cases have been underreported in areas where the disease is highly endemic. However, the range of probabilities in our model allows for both underreporting and overdiagnosis.

The benefits are likely highest if both community-level incidence of Lyme disease and individual risk for exposure to tick bites and infection (38) can be considered in using the vaccine. The Advisory Committee on Immunization Practices, Public Health Service, U.S. Department of Health and Human Services, recently agreed with this conclusion and voted, in February 1999, to recommend the use of Lyme disease vaccine on the basis of a combination of both community-level and individual risk. These recommendations will be published soon (49).

Ours is not the only study to suggest that the vaccine not be used universally. A forthcoming Institute of Medicine report (50) uses cost per quality-adjusted life year (QALY) saved to examine vaccine priorities. The authors estimate that it would cost more than $100,000 per QALY saved if the vaccine were given ". . . to resident infants born in, and immigrants of any age to, geographically defined high risk areas." This result led the authors to rank Lyme disease vaccine as "less favorable," their lowest ranking in terms of priorities for vaccine development.

Our model also considers the relative value of two interventions: vaccination and the detection and treatment of early Lyme disease. Communities with average individual probabilities of contracting Lyme disease of less than 0.01 may benefit from interventions that improve the probability of early diagnosis and treatment of Lyme disease.

Dr. Meltzer is senior health economist, Office of the Director, National Center for Infectious Diseases, CDC. His research interests focus on assessing the economics of public health interventions such as oral raccoon rabies vaccine, Lyme disease vaccine, and hepatitis A vaccine, as well as estimating the economic cost of bioterrorism, dengue, pandemic influenza, and other infectious diseases. His research uses various methods, including Monte Carlo modeling, willingness-to-pay surveys (contingent valuation), and the use of nonmonetary units of valuation, such as disability adjusted life years.

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Use of Antimicrobial Growth Promoters in Food Animals and *Enterococcus faecium* Resistance to Therapeutic Antimicrobial Drugs in Europe

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Supplementing animal feed with antimicrobial agents to enhance growth has been common practice for more than 30 years and is estimated to constitute more than half the total antimicrobial use worldwide. The potential public health consequences of this use have been debated; however, until recently, clear evidence of a health risk was not available. Accumulating evidence now indicates that the use of the glycopeptide avoparcin as a growth promoter has created in food animals a major reservoir of *Enterococcus faecium*, which contains the high level glycopeptide resistance determinant *vanA*, located on the Tn*1546* transposon. Furthermore, glycopeptide-resistant strains, as well as resistance determinants, can be transmitted from animals to humans. Two antimicrobial classes expected to provide the future therapeutic options for treatment of infections with vancomycin-resistant enterococci have analogues among the growth promoters, and a huge animal reservoir of resistant *E. faecium* has already been created, posing a new public health problem.

Vancomycin-Resistant *Enterococcus faecium* (VRE)

In addition to being a member of the normal gut flora of nearly all warm-blooded animals (including humans), *E. faecium* has the ability to cause a wide range of infections, primarily serious infections in hospital patients (particularly in intensive care units). Increasing incidence of *E. faecium* infections has been associated with use of third-generation cephalosporins in hospitals (1). Enterococci are resistant to many antibiotics. In an increasing number of cases, vancomycin is the only treatment drug that remains effective. Because *E. faecium* was untreatable with practically all other antibiotics, the emergence of the first high level vancomycin-resistant *E. faecium* was of particular concern (2). By 1997, more than 15% of nosocomial enterococcal infections in U.S. hospitals were due to VRE (3).

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The vancomycin-resistant strain of *E. faecium* (VRE) contains the VanA gene cluster located at a mobile genetic element, a transposon designated Tn*1546*. Although other mechanisms and determinants of glycopeptide resistance have been found in *E. faecium*, in this article, VRE will refer to strains containing the *vanA* gene and Tn*1546*. First isolated in France in 1986, VRE were subsequently found in the United States in 1989, where they rapidly became a frequent cause of hospital infections (3). Like many other nosocomial pathogens, VRE were believed to originate and be maintained in hospitals and to have little, if any, association with the community. Nosocomial outbreaks and clonal spread in the United States supported this assumption (4). In Europe, however, even though serious incidents have occurred in some countries, VRE-associated hospital infections have not increased at the same rate and to the same proportion as in the United States (5). The first indication that the epidemiology of VRE may differ from that of other gram-positive organisms capable of causing hospital infections (e.g., methicillin-resistant *Staphylococcus aureus*)

came from the United Kingdom, where Bates et al. (6) reported isolating VRE from pig herds as well as from the farm environment. These scientists suggested that a community source of VRE may exist. Soon afterwards, Klare et al. (7) in Germany showed that VRE could be cultured frequently from pigs, poultry, and humans in the community and suggested that VRE may be associated with the use of glycopeptides as growth promoters in food animals.

Antimicrobial Growth Promoters

Antimicrobial growth promoters (AGPs) are antibiotics added to the feed of food animals to enhance their growth rate and production performance. The mechanism by which AGPs work is not clear. AGPs reduce normal intestinal flora (which compete with the host for nutrients) and harmful gut bacteria (which may reduce performance by causing subclinical disease). The effect on growth may be due to a combination of both fewer normal intestinal flora and fewer harmful bacteria. The class of antimicrobial drugs used and the animal species involved may determine the relative importance of each mechanism (8). The quantity used in feed varies with each antimicrobial agent. In the European Union (EU), avoparcin 20 mg/kg and 40 mg/kg was approved for different age groups of pigs and chickens; the concentration is often referred to as "subtherapeutic" (not to be confused with sub-MIC levels). The resulting concentration in the gastrointestinal tract of the animal is sufficient to inhibit the susceptible bacteria and markedly affect the composition of the bacterial gut flora (8).

In Denmark, as well as in other countries, only a few glycopeptides have been used; for humans vancomycin and (to a lesser extent) teicoplanin have been used, and for animals avoparcin has been used exclusively as a feed additive for growth promotion. Avoparcin has not been used in animals in Sweden since 1986 because of a national prohibition of AGPs; in the United States, avoparcin was never approved because of its carcinogenic effects (9).

Few countries have accurate data on the use of antibiotics in animals and humans. In Denmark, 24 kg of active vancomycin was used for human therapy in 1994. In comparison, 24,000 kg of active avoparcin was used as a feed additive for animals (10). In Austria, an average of 582 kg of vancomycin was imported for medical purposes and 62,642 kg of avoparcin for animal husbandry per year from 1992 to 1996 (11). Thus, although there are more food animals than humans, the selective pressure favoring VRE in Europe can be estimated to be much higher in food animals than in humans. Data on the yearly use of vancomycin in the United States and in major European countries were recently published by Kirst et al. (12). Denmark, a small country, used more glycopeptide growth promotant (avoparcin) than all of Europe and the United States used for treating ill humans (vancomycin). Difference in denominators implies huge difference in use (10).

Use of Avoparcin as a Growth Promoter and the Occurrence of VRE in Food Animals

The high selective pressure by the use of glycopeptides as growth promoters could explain the presence of VRE in food animals. We have conducted a number of studies to investigate the association between the use of avoparcin as a growth promoter and the occurrence of VRE.

In one study, eight poultry flocks raised conventionally and six raised without growth promoters were compared (13). No VRE was found in birds raised without growth promoters, whereas five out of eight conventional flocks contained VRE. Isolation rates in positive flocks were as follows: of five fecal samples tested, one to four (20%–80%) were positive. Twenty-two pig herds and 24 poultry flocks, half of which had used avoparcin and half of which had not, were compared by occurrence of VRE in fecal samples collected from animals of the herds and flocks. A strong and statistically highly significant association between the presence of VRE and the use of avoparcin was observed (14). Of 12 pig herds using feed with avoparcin, 8 had VRE, while of 10 herds not using avoparcin, 2 had VRE ($p = 0.043$, risk ratio [RR] 3.3; 95% confidence interval [CI]: 1.1, 10.0). In broiler farms where avoparcin was used, VRE was isolated from 11 of 12 fecal samples. In farms where avoparcin was not used, VRE was isolated in 2 of 12 samples ($p < 0.0006$; RR 5.5; 95% CI: 2.2, 13.9).

The association observed at the flock and herd level has also been observed at the country level. In countries where avoparcin had been used as a growth promoter, VRE could frequently be cultured from food animals, whereas in countries where avoparcin had not been used, VRE were not detected (Table 1). These

findings are consistent with the hypothesis that use of avoparcin has created a reservoir for VRE in food animals.

Table 1. Avoparcin as a growth promoter in countries where the occurrence of vancomycin-resistant enterococci (VRE) in animal husbandry has been investigated

Country	Avoparcin used	VRE in animal husbandry	Ref.
Belgium	+	+	15
Denmark	+	+	13,14
Finland	+	+	16
France	+	+	17
Germany	+	+	7
Great Britain	+	+	6
The Netherlands	+	+	18
Norway	+	+	19
Sweden	-	-	20
United States	-	-	21,22

Transmission of VRE from Animals to Humans

Can an animal reservoir in itself be regarded a public health risk? What are the chances that VRE or the resistance genes will be transmitted from animals to humans? A public health risk must be assumed to exist when transfer from animals to humans can be shown directly or indirectly.

VRE are frequently present in food produced in Denmark as well as in food imported into Denmark from other European countries (23,24). Thus, exposure to humans from insufficiently heated food or cross-contaminated ready-to-eat food takes place. Unlike studies in the United States (21,25), European studies reported that humans frequently are fecal carriers of VRE (26-29). This suggests that VRE can be ingested from food in Europe. Furthermore, VRE was not detected in strict vegetarians in The Netherlands, supporting the view that the source of VRE is contaminated meat (Table 2) (28).

Table 2. Prevalence of vancomycin-resistant *Enterococcus faecium* in fecal samples of residents in a vegetarian and a nonvegetarian nursing home, The Netherlands (28)

	No. persons investigated	No. *E. faecium* positive	No. VRE positive[a,b]
Vegetarians	42	23	0
Nonvegetarians	62	32	6

[a]$P<0.05$.
[b]All VRE-positive samples were *E. faecium*.

Molecular typing shows a very high diversity of VRE types in animals as well as humans (30). Nevertheless, similar or related types have been shown to occur in animals and humans on a number of occasions, supporting the assumption that transfer of VRE between humans and animals does take place (18,19). We have recently compared 84 isolates of *E. faecium* from swine, chickens, and humans in Denmark by *Sma*I generated macrorestriction profiles and *Eco*RI ribotyping. Similarity analysis by unweighted pair group method with arithmetic averages–derived dendrograms did not indicate a higher degree of similarity among *E. faecium* isolates (VRE as well as non-VRE) from humans than from animals. This finding indirectly supports the hypothesis that *E. faecium* from different food animals and humans are not discrete populations but belong to a common pool of *E. faecium* shared by animals and humans (data not shown).

The *VanA* gene cluster encoding for vancomycin resistance in animal and human VRE is located on a transposon designated Tn*1546* (31,32). Tn*1546* can easily spread from one enterococcal species to another as well as from enterococci to *S. aureus* (33,34). Recent investigations have documented that in vivo transfer of Tn*1546* can take place in the mammalian intestinal tract (A. Sundsfjord, pers. comm.). Furthermore, animal VRE can colonize the human intestinal tract for at least 3 weeks after experimental ingestion of 10^7 CFUs of a single strain (35). This indicates that vancomycin resistance can spread in the gastrointestinal tract from transiently colonizing animal VRE to *E. faecium* strains of the resident human gut flora.

The *VanA* gene cluster consists of several genes. We investigated the genes and the regions between them by sequencing of selected areas, polymerase chain reaction amplification of other areas, and hybridization with specific probes (36,37). Thirteen different types were observed. Most differences arose from the presence of insertions or deletions in noncoding intergenic regions. One nucleotide difference was observed in the coding sequences; this point mutation occurred in the *vanX* gene at position 8234, where a G in the reference VRE strain was substituted for a T in some isolates.

In human VRE isolates, this mutation was evenly distributed, whereas in poultry isolates from different countries only the G variant

occurred; in isolates from swine from different countries the T variant occurred in nearly all isolates (Table 3). Although we have no explanation for the uneven distribution of subtypes between different animals, the finding of both types in humans does support the hypothesis that animals are a primary source of vancomycin resistance genes in humans, whereas humans apparently do not serve as reservoir for animals, in which case both types would be expected to occur in both animal species. In the same investigation, we found that all human isolates from a Muslim country belonged to the poultry subtype (37). The absence of pork variant types in a Muslim country suggests that food of animal origin is a major reservoir for VRE in humans.

Table 3. Variations in Tn*1546*-like elements of vancomycin-resistant *Enterococcus faecium* isolates of animal and human origin (37)

Source	No. isolates	T variant	G variant
Humans	45	16	29
Pigs	33	32	1
Poultry	193	0	193
Total	271	48	223

The European/American Paradox

Even though the greater frequency of VRE infections in U.S. than in European hospitals would seem to contradict it (12), the hypothesis that animals could serve as reservoirs of human VRE infections is supported by several lines of indirect evidence.

Heavy use of vancomycin (and probably also third-generation cephalosporins) is a prerequisite for frequent VRE infections in hospitals. Heavy use of vancomycin and third generation cephalosporins is more frequent in U.S. than in European hospitals (12,18). Thus, the problem in Europe, irrespective of a high carrier rate of VRE in the community, has not grown to the same proportions as in the United States. VRE infections in the United States are probably due to the heavy use of antibiotics in hospitals and the eventual spread of VRE within and among hospitals by carrier personnel (38).

Another prerequisite for high incidence of VRE hospital infections is a source of VRE. In the United States, primary sources of VRE to hospitals include travelers returning from abroad, tourists, and imported food. Once inside the hospital, VRE can cause nosocomial outbreaks because of its high potential to colonize and persist in the environment, which facilitates its persistence and spread (4). An alternative hypothesis could be that some clones of VRE have a higher potential to cause infections and that such clones are more prevalent in United States. This hypothesis, however, is contradicted by the high number of different types of VRE causing infections in Europe and the United States, which suggests that pathogenic potential is not limited to a few clones of VRE (38).

Avoparcin was approved for growth promotion in Europe in 1974. The first VRE was detected in a human patient in France in 1986. Does this suggest that VRE was not present in animals before 1986? Historically, resistance to growth promoters in animal bacteria was not monitored, and with few exceptions the studies conducted have looked at bacteria other than enterococci because enterococci were not considered foodborne pathogens. Thus, if VRE were not detected in food animals, it may be because they were not looked for.

The Effect of Prohibiting Use of Avoparcin as a Growth Promoter

Because of public health concerns about resistance to glycopeptide antibiotic drugs, avoparcin was banned in Denmark in 1995. In 1996, Germany took a similar step, and finally in 1997, avoparcin was banned in all EU member states. After the ban in Denmark, a marked reduction in the occurrence of VRE in Danish poultry flocks has been observed at slaughter (from 82% in 1995 to 12% of flocks in 1998; $x^2 = 68.3$ on 5 df.; $p < 0.0001$), whereas in swine only a minor reduction has been observed (Figure) (39). In Germany, a decrease in the incidence of VRE in poultry meat and in fecal samples from humans in the community was observed after discontinuation of avoparcin use in animal husbandry (40). In poultry meat the proportion of VRE-positive samples were reduced from 100% in 1994 to 25% in 1997, and in fecal samples from humans in the community, the carrier rate decreased from 12% in 1994 to 3% in 1997.

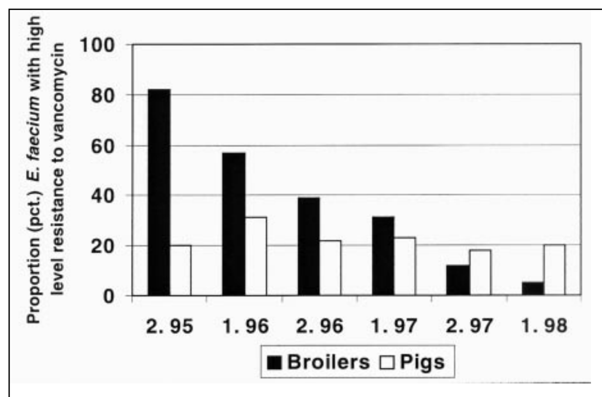

Figure. Trend in the proportion of *Enterococcus faecium* isolates resistant to vancomycin (VRE) during successive half-year periods from second half of 1995 to first half of 1998 (39).

Similar Problems Related to Other Antimicrobial Growth Promoters

Most of the different growth promoters approved in the EU are active against gram-positive bacteria. With increasing resistance in gram-positive pathogenic bacteria, antimicrobial drugs used as growth promoters have attracted renewed attention as potentially useful for human therapy. More than 10 years after the first VRE was discovered, the first drug for humans with good clinical effect against VRE infections is ready to be marketed. This drug is a combination of two streptogramins, quinupristin and dalfopristin (Synercid).

For decades, virginiamycin, which belongs to the group of streptogramins, has been used as a growth promoter in the European Union as well as in the United States, primarily for poultry production. Investigations in the United States, The Netherlands, and Denmark have frequently found Synercid-resistant *E. faecium* in poultry (41-43). As for VRE, we have no data on the prevalence of streptogramin-resistant *E. faecium* in animal husbandry before virginiamycin was used as a growth promoter. No monitoring has been carried out. Moreover, the gene (*satA*) conferring resistance to virginiamycin and Synercid have been found in animals and humans (44), and in vivo transfer of these genes from resistant to sensitive strains of *E. faecium* in the mammalian gastrointestinal tract has been shown (45). Thus, the events associated with avoparcin and vancomycin may be recurring for Synercid and virginiamycin. Furthermore, the drug anticipated to be next in line after Synercid, a compound called Ziracin, belonging to the class of everninomicins, is practically identical to another growth promoter called avilamycin, which has primarily been used in poultry.

We have detected avilamycin resistance in 69% of *E. faecium* isolates from poultry in Denmark (43). Moreover, preliminary investigations show that resistance to avilamycin gives cross-resistance to Ziracin and that a transferable genetic element may be involved (46). Thus, again use of an antimicrobial drug as a growth promoter may have created a major animal reservoir of resistant *E. faecium,* threatening to shorten the life span of a new promising drug when it is put to use in humans.

Future Perspectives

At the core of the VRE issue appears to be the way antimicrobial drugs are being developed. New classes of antimicrobial drugs are not available. Instead, old drugs are being modified that may have been used in agriculture as growth promoters for decades because they were not considered useful for humans. Now that physicians are searching for more options in antibiotic treatment, the older drugs may no longer be viable. The use of antimicrobial drugs and development of resistance in animals and humans are interrelated. Therefore, systems to monitor antimicrobial resistance in pathogenic and commensal bacteria should be established. Such systems should cover relevant bacteria from the entire farm-to-fork chain and monitor resistance towards antimicrobial drugs used in both animals and humans, including growth promoters (47-49).

Finally, antimicrobial agents should not be used for growth promotion if they are used in human therapeutics or are known to select for cross-resistance to antimicrobial drugs used in human medicine (47). Antimicrobial agents are too valuable to be used as a tool in animal production because any antimicrobial drug may be useful for human therapy in the future even if not used therapeutically today. Adherence to the World Health Organization recommendations (47) will ensure a systematic approach toward replacing antimicrobial growth promoters with safer nonantimicrobial drug alternatives. The EU countries entered this process in December 1998 when four growth promoters (tylosin, spiramycin, bacitracin, and virginiamycin) were banned because of their structural relatedness to

therapeutic antimicrobial drugs used for humans (50).

Dr. Wegener is head of The Danish Zoonosis Centre. His research interests are veterinary public health, microbiology, epidemiology, molecular biology; control of zoonoses, particularly the so-called "modern" bacterial zoonoses; and potential public health consequences of the use of antimicrobial drugs in animal husbandry.

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Bacterial Vaccines and Serotype Replacement: Lessons from *Haemophilus influenzae* and Prospects for *Streptococcus pneumoniae*

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Conjugate vaccines have reduced the incidence of invasive disease caused by *Haemophilus influenzae*, type b (Hib), in industrialized countries and may be highly effective against *Streptococcus pneumoniae*. However, the serotype specificity of these vaccines has led to concern that their use may increase carriage of and disease from serotypes not included in the vaccine. Replacement has not occurred with the use of Hib vaccines but has occurred in trials of pneumococcal vaccines. Mathematical models can be used to elucidate these contrasting outcomes, predict the conditions under which serotype replacement is likely, interpret the results of conjugate vaccine trials, design trials that will better detect serotype replacement (if it occurs), and suggest factors to consider in choosing the serotype composition of vaccines.

Conjugate vaccines are a major advance in the control of diseases caused by two members of the normal bacterial flora of the human nasopharynx, *Streptococcus pneumoniae* (pneumococcus) and *Haemophilus influenzae* type b (Hib). In the absence of widespread vaccination, pneumococci have been responsible for an estimated 7 million cases of otitis media, 500,000 cases of pneumonia, 50,000 cases of bacteremia, and 3,000 cases of meningitis each year in the United States (1). Before the widespread use of conjugate vaccines, Hib caused invasive disease in an estimated 1 in 200 children <5 years of age in the United States (2). Conjugate vaccines have reduced the incidence of invasive Hib disease by 90% or more in industrialized countries (2,3). After promising phase-II clinical trials (4-7), the first results from a phase-III trial of a pneumococcal conjugate vaccine have shown very high efficacy against invasive disease (8).

In addition to protecting against disease, conjugate vaccines protect against asymptomatic carriage of the target organisms (4-7,9). *H. influenzae* and *S. pneumoniae* are frequently found in the normal nasopharyngeal flora of healthy persons, with invasive disease being relatively rare compared with asymptomatic carriage (10,11). Asymptomatic carriers are also responsible for most transmission of these organisms (10,11); in contrast to many other vaccine-preventable infections, disease caused by these organisms seems to contribute little to the process of transmission (12-14). By reducing the rate of carriage of targeted bacteria, conjugate vaccines also reduce their transmission and should thereby offer protection to unvaccinated contacts of vaccinated persons. It has been shown (in the case of Hib) and suggested (in the case of pneumococcus) that the use of conjugate vaccines results in herd immunity (15). Herd immunity may explain why the reduction in invasive Hib disease in some populations has exceeded the fraction of the population that received the vaccine (3) and why Hib invasive disease declined even in age groups that had not yet received the vaccine (16).

Although the reduction in carriage achieved by conjugate vaccines is beneficial from the perspective of herd immunity, it has raised concerns about the possibility of serotype replacement. Both *H. influenzae* and pneumococci are characterized by extensive antigenic diversity in their polysaccharide capsules. In

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H. influenzae, six capsular types are known, in addition to a large group of nontypeable (unencapsulated) variants. Before vaccination, serotype b was responsible for most invasive diseases, with minor contributions from the other encapsulated types. Because of the importance of Hib in invasive disease, vaccination efforts have concentrated on the b serotype (16). Pneumococci are even more diverse, with 90 recognized serotypes; many of these serotypes are capable of causing invasive disease. To accommodate this greater diversity, pneumococcal conjugate vaccines have incorporated multiple serotypes. Because the protection offered by conjugate vaccines is specific to the capsular type(s) included in the vaccine, it has been suggested that reducing carriage of these vaccine types may leave open an ecologic niche that will be filled by serotypes not included in the vaccine (5,17-20).

Hib conjugate vaccines served as a model for the development and testing of pneumococcal vaccines. However, pneumococci are epidemiologically different from Hib, and results of clinical trials with pneumococcal conjugates suggest that the two bacteria differ in their response to vaccination, especially with respect to serotype replacement. This article describes how mathematical models can be used to elucidate these contrasting outcomes, specify the conditions under which serotype replacement is likely, interpret the results of conjugate vaccine trials, design trials that will be better able to detect serotype replacement (if it occurs), and suggest factors to consider in choosing the serotype composition of vaccines.

Serotype Replacement: Hib and Pneumococcal Conjugate Vaccines

Serotype replacement has not been detected since the introduction of Hib conjugate vaccines. Studies of *H. influenzae* carriage in 700 children in Finland (21) and 364 families in the United Kingdom (15,22) found no evidence of increased carriage of non-b *H. influenzae* as a result of vaccination. Although increases in invasive disease from other nasopharyngeal bacteria have been reported since Hib vaccination began (23,24), no evidence of a causal link to Hib vaccination has been observed (22,23). Furthermore, a recent study in the United States showed that the net impact of Hib vaccination has been a 68% reduction in invasive disease from all *H. influenzae* between 1986 and 1995 (25); therefore, any increase in disease from non-b serotypes is small compared with the reduction in disease from type b.

In contrast, pneumococcal conjugate vaccine studies show considerable evidence of serotype replacement, as measured by nasopharyngeal carriage of nonvaccine type organisms. Increases in the carriage of nonvaccine serotypes have occurred in three major ongoing clinical trials of pneumococcal conjugate vaccines. In Gambia, carriage of nonvaccine serotypes was 79% in children receiving three doses of a pneumococcal conjugate vaccine (compared with 42.5% in controls) (5). In trials of a 9-valent vaccine in South Africa, carriage of nonvaccine serotypes increased from 21% in controls to 39% in vaccine recipients (6). Serotype replacement was observed in the second of two large studies in Israel (4,7); the reason for the difference in outcome between the two studies remains unclear. In the first phase-III trial for which data were presented, no increase was observed in invasive disease from nonvaccine types (8). While this result is encouraging, it may not be indicative of what will occur as conjugate vaccines enter widespread use in a variety of communities.

A Mathematical Model of Vaccination against Colonizing Bacteria

Mathematical models can be useful in defining the extent of serotype replacement in various contexts, optimizing the design of clinical trials to discern whether such replacement occurs, and interpreting the results of these trials. With these goals in mind, I constructed and analyzed a mathematical model of the transmission dynamics of colonizing bacteria with multiple serotypes, such as pneumococci, and the effect of vaccination on these dynamics. The model is similar in structure to the compartmental models used to design and predict the effects of vaccination programs against other infectious diseases (26).

The main distinguishing feature of this model is that it simultaneously considers the transmission of two (or more) strains of the same organism. The model is designed to analyze the effects of competitive interactions between these strains, in which carriage of one serotype reduces the probability that a host will be colonized with another serotype. If such

competitive interactions occur, serotype replacement is possible, because vaccine-induced reductions in some serotypes will increase the opportunities for others to spread in the population. Epidemiologic studies have provided indirect evidence of such competitive interactions (27-29), while laboratory studies have suggested mechanisms by which different species of streptococci (30,31) or different strains of *H. influenzae* (32) might compete in the nasopharynx. At present, however, little is known about the precise nature of these interactions, and perhaps the most compelling evidence that competition occurs comes from the replacement observed in pneumococcal conjugate vaccine studies.

The assumptions and structure of the model are as follows. In the absence of vaccination, the model (Figure 1) assumes that humans are born into the susceptible (X) compartment at a particular rate and are removed from that compartment (and all other compartments) at a specific per capita death (or maturation) rate. Two pneumococcal serotypes (designated 1 and 2) are present, and susceptible hosts may be colonized by either type; colonization moves the host into the Y_1 or Y_2 compartment, respectively. The incidence of colonization with each type is proportional to the total number of persons carrying that type. Colonization has average duration $1/\gamma$. While carrying one serotype, a host may be colonized by the other type, which moves the host into the dually colonized compartment (Y_{12}). This secondary colonization also occurs at a rate proportional to the prevalence of the colonizing type, but a rate that is c_j (j = 1 or 2) times the rate at which a susceptible person would be colonized by the same type. Thus, c_j is an inverse measure of the competitive inhibition of type j by the resident type in a host.

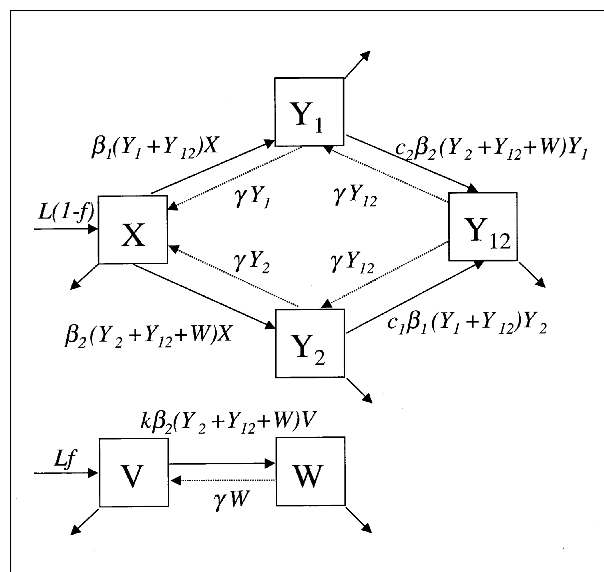

Figure 1. The structure of the mathematical model described in the text and in greater detail (30).

When vaccination begins, a fraction f of all persons are assumed to be vaccinated at birth. In the model, these persons are born into the vaccinated (V) compartment. It is assumed that vaccination completely protects a person against carriage of type 1 (this is done to simplify the analysis of the model; if only partial protection were offered, the effects would be similar to those observed at a lower level of vaccine coverage f). To consider the effects of including more than one bacterial serotype in the vaccine, the model can accommodate vaccines that are effective only against type 1 (monovalent vaccines), as well as those that give either partial or full protection against type 2 (bivalent vaccines). The parameter k represents the degree of protection offered by the vaccine against serotype 2.

By varying the parameters of the model, it is possible to compare the effects of different levels of vaccine coverage (fractions of the population vaccinated), different assumptions about the competitive interactions among pneumococcal serotypes, and different types of vaccines (monovalent vs. bivalent) (33). In summary, the major predictions of the model are as follows.

1) If there is competition between different pneumococcal serotypes to colonize hosts, vaccination against serotype 1 alone will increase the prevalence of serotype 2. The extent of replacement, measured as the increase in the prevalence of serotype 2, will be greatest when vaccine coverage is high and when serotype 2 is strongly inhibited from colonizing persons who carry serotype 1. Serotype replacement may take either of two forms: an increase in prevalence of a type already present in the population or the appearance and spread of types previously absent from the population because they were unable to compete with the vaccine type(s).

2) Bivalent (or polyvalent) vaccines can also cause replacement if the protection offered against different serotypes is uneven. In particular, if a vaccine has relatively low efficacy

against serotype 2 but very high efficacy against serotype 1, use of a bivalent vaccine may increase the prevalence of type 2.

3) If only two serotypes interact in a population, the amount of replacement that can occur is limited. Specifically, the increase in the prevalence of serotype 2 will always be less than or equal to the decrease in the prevalence of serotype 1. Thus, for example, if the prevalences of serotypes 1 and 2 before vaccination are 15% and 20%, respectively, then the prevalence of serotype 2 after vaccination will be no more than 35%.

4) If more than two types are competing to colonize hosts, this limitation need not hold. In the presence of more than two types, vaccination can increase the prevalence of a single, nonvaccine type more than it reduces the prevalence of the vaccine type.

5) Although replacement is of concern, it may also be beneficial. If serotypes compete to colonize hosts, increases in the prevalence of the nonvaccine types will help reduce the prevalence of the serotypes included in the vaccine. Thus, replacement will augment the effects of herd immunity in reducing the exposure of all members of the population to vaccine serotypes. This results in a tradeoff between the breadth of coverage of a vaccine (number of serotypes covered) and the effectiveness of the vaccine in reducing carriage of each serotype at the population level.

The model's predictions have several implications for the interpretation of existing data from the use of conjugate vaccines, the design of vaccine trials, and the choice of vaccine composition.

Why Has Replacement Carriage Occurred with Pneumococcal Conjugate Vaccines but Not with Hib Vaccines?

As noted above, the absence of serotype replacement observed with the use of Hib in industrialized countries contrasts with the findings of considerable serotype replacement in two studies of pneumococcal vaccines. What might account for this difference?

The mathematical model suggests an explanation. The model predicts that, in a pairwise interaction between two serotypes, the increase in prevalence of a nonvaccine type will be no more than the reduction in prevalence of a vaccine serotype. This principle is illustrated in Figure 2, which presents data from a study of Hib conjugate vaccine in the United Kingdom (15). In the figure, the white bars show the prevalence of each of three *H. influenzae* serotypes—b, e, and f—in vaccinated persons, and the black bars show the prevalence of each of these serotypes in controls. If one assumes that Hib interacts independently with each of the two nonvaccine serotypes (e and f), one can use the two-serotype model to calculate the maximum prevalence of these nonvaccine types in vaccinees that would be expected if these serotypes compete very strongly with serotype b. The striped bars show the maximum prevalence of types e and f expected in the study, where only a small fraction of the community was vaccinated; the shaded bars indicate the equivalent figure if the whole community had been vaccinated. As is clear from the figure, the increase in nonvaccine-type carriage in vaccinees would be minuscule and statistically undetectable in a study of this kind (indeed, the study from which these data were drawn was not designed to detect replacement; data on the prevalence of types e and f were used to control for general changes in

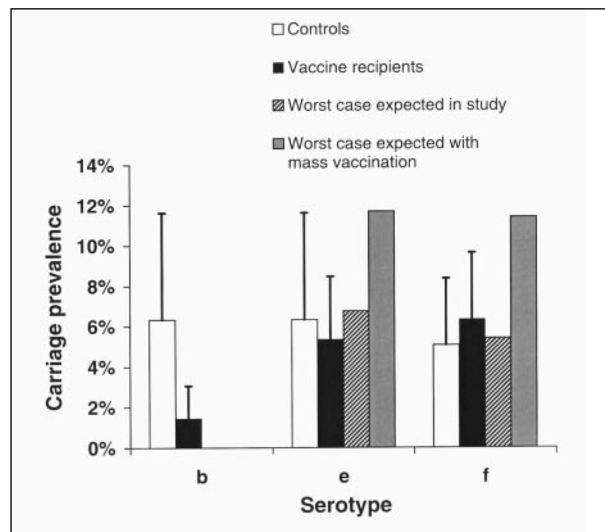

Figure 2. Carriage of three serotypes of *Haemophilus influenzae* in children vaccinated against serotype b (white bars) and in controls (black bars) (14). Error bars indicate 95% confidence interval (binomial approximation). Shaded bars show the maximum carriage of serotypes e and f in vaccine recipients that could result from replacement in a population where only a small proportion of susceptibles are vaccinated (as in the study). Striped bars show the equivalent figures in a hypothetical study in which virtually all susceptibles were vaccinated.

the prevalence of *H. influenzae* that could have been attributable to factors other than vaccination [15]). The reason is that the prevalence of Hib was so low before vaccination that even its complete removal by widespread vaccination would have little effect on competing bacteria. The prevalence of Hib carriage in other industrialized countries is similar to that measured in the UK study. Therefore, the model suggests that the lack of replacement, even after widespread use of the Hib conjugate vaccine in industrialized countries, may be a simple result of the low prevalence of Hib carriage.

If this interpretation is correct, then serotype replacement would be more likely to occur in areas where the prevalence of Hib is higher or for vaccination against other organisms whose prevalence is higher. This difference could account for the contrasting outcomes of vaccination against Hib and pneumococci. Differences in the biology of colonization or in the interactions between bacterial types may also have a role in these contrasting outcomes. Distinguishing the relative importance of these two explanations will require further research into the biologic interactions of bacterial populations in the nasopharynx, as well as studies of the effects of conjugate Hib vaccination in areas where Hib's prevalence is higher.

Detection of Replacement: The Design of Clinical Trials

If used by a large fraction of the human population in a community, a conjugate vaccine may alter the composition of the bacterial population, not only in vaccinated, but also in unvaccinated persons in that community. Vaccination may reduce the prevalence of serotypes included in the vaccine, thereby protecting unvaccinated persons against exposure to these serotypes (herd immunity). Similarly, if serotype replacement occurs and vaccinated persons become more likely to carry nonvaccine serotypes, the exposure of unvaccinated persons to these serotypes will increase. As a result of these indirect effects, strain replacement will be magnified in communities where large numbers of persons are vaccinated.

This process is also evident from Figure 2. There, the striped bars show the model's prediction of the maximum increase in nonvaccine type carriage in vaccine recipients in a community in which the vaccine is used only on a very small proportion of the population, while the shaded bars show the same increase in a community where everyone is vaccinated. As is clear from the figure, replacement will be most easily observed in communities where the level of vaccine coverage is high.

Therefore, one would expect that the extent of serotype replacement when vaccines enter widespread use in a community may be much greater than that observed in clinical trials where a relatively small fraction of the community is immunized. This is one important reason why the failure to observe an increase in invasive disease from nonvaccine-type pneumococci in the Northern California trial (8), while promising, may not be indicative of the potential for replacement once the vaccine is used on a large scale. If one is interested in designing a clinical trial that simulates the selective pressures exerted by communitywide use of a conjugate vaccine, and therefore maximizes the chances of observing serotype replacement during the trial, then community-randomized clinical trials will be superior to individually randomized ones. Studies of pneumococcal vaccines in which communities are the units of randomization are under way in Native American communities in the southwestern United States (K. O'Brien, pers. comm.).

Vaccine Composition: Replacement Revisited

For an organism like pneumococcus, in which a number of serotypes can cause disease, the choice of serotypes for inclusion in a conjugate vaccine is critical. One strategy would be to include as many serotypes as possible to achieve the broadest possible protection. In addition to some clinical limitations on the number of serotypes that can be included in a single vaccine, there are other reasons why such a strategy would not be ideal. As noted above in the last prediction from the model, serotype replacement can augment the effectiveness of a vaccination program in a community. This occurs because increases in the prevalence of nonvaccine serotypes competitively inhibit carriage of vaccine serotypes. Ideally, then, one would like to design a vaccine that maximizes these beneficial effects while minimizing the risk of added disease from increased carriage of nonvaccine serotypes.

The question is how to accomplish such a balance. So far, the model describes only carriage of various serotypes; it does not directly address the problem of disease. The effect of vaccination on disease will depend both on changes in patterns of carriage of different serotypes and on the propensity of the individual serotypes to cause disease. Serotypes of *H. influenzae* and *S. pneumoniae* vary considerably in their pathogenicity, as manifested by experimental evidence (34) and by differences between the frequency of particular serotypes in carriage isolates and their frequency in disease isolates (17,35). If these serotype associations were stable, the ideal vaccine could simply include the most pathogenic serotypes but exclude those that tend to be avirulent, thereby taking advantage of any increases in the prevalence of the avirulent serotypes to augment the effect of the vaccine (36).

This approach has several limitations. First, the model predicts that widespread use of a vaccine may result in the appearance of bacterial types which, before vaccination, had been absent from the population because of competition from vaccine types. The virulence of these novel types would be difficult to predict, since competitive inferiority to existing types need not be correlated with low virulence (12,13). Second, both species discussed here are highly transformable. Although capsular type seems to be very closely associated with virulence in *H. influenzae* (34,37), transformation studies in pneumococci have shown complicated interactions between capsular type and other genes in determining virulence (38), so the existing associations between virulence and capsular type in pneumococci (39,40) may change in response to conjugate vaccine-induced selective pressure. If such vaccines are used on a widespread scale, surveillance of shifts in the serotype associations of invasive disease should be maintained.

Serotype replacement has been discussed primarily as it applies to serotypes not included in the vaccine. However, if the vaccine is only weakly effective in immunizing against carriage of some of the serotypes included in it, even these serotypes may increase in prevalence after vaccination is introduced. This can occur if the efficacy of the vaccine against these serotypes is outweighed by its effect in removing competing serotypes. Results of trials published thus far indicate that the protection offered by the vaccine against included serotypes taken together is considerably lower than 100%. Therefore, the results of future trials should be monitored to determine whether prevalence of any of the individual vaccine serotypes is increasing in vaccinated hosts.

Interpreting Replacement: Is It Real?

Studies of pneumococcal carriage are typically performed by sampling the nasopharyngeal flora of vaccinated and unvaccinated persons, plating the samples on agar, and serotyping one or a few colonies. This technique typically identifies the most abundant pneumococcal serotypes carried by a person, and possibly a minority type if it is present in large numbers. However, many people carry more than one pneumococcal type (27,41), and when the pneumococci are studied in detail, the minority type may be much less plentiful than the majority type—at a frequency of 10% or less (41). Therefore, current methods are likely to have very low sensitivity for the detection of minority types.

This creates a problem in measuring serotype replacement during pneumococcal vaccine trials. Vaccinated persons, who are protected against carriage of vaccine types, may become more susceptible to carriage of pneumococcal types not included in the vaccine. This is serotype replacement, a phenomenon that vaccine trials are intended, in part, to detect. In addition, nonvaccine type pneumococci, even if they are not more plentiful, may be more readily detected in vaccinated persons. Some unvaccinated persons carry both vaccine-type and nonvaccine-type pneumococci, and in some of them, the vaccine-type will be in the majority. Because minority populations of pneumococci are difficult to detect, the nonvaccine-type pneumococci carried by these persons is masked by the vaccine types, resulting in an underestimate of the prevalence of nonvaccine-type pneumococci in the unvaccinated population. Vaccinated persons, by contrast, are less likely to carry vaccine-type pneumococci, so their nonvaccine-type pneumococci are more likely to be detected. This is known as unmasking. Figure 3 illustrates the distinction between serotype replacement and unmasking. Unmasking is an artifact of sampling, and one would like to be able to determine whether a finding of higher nonvaccine-type carriage rates in

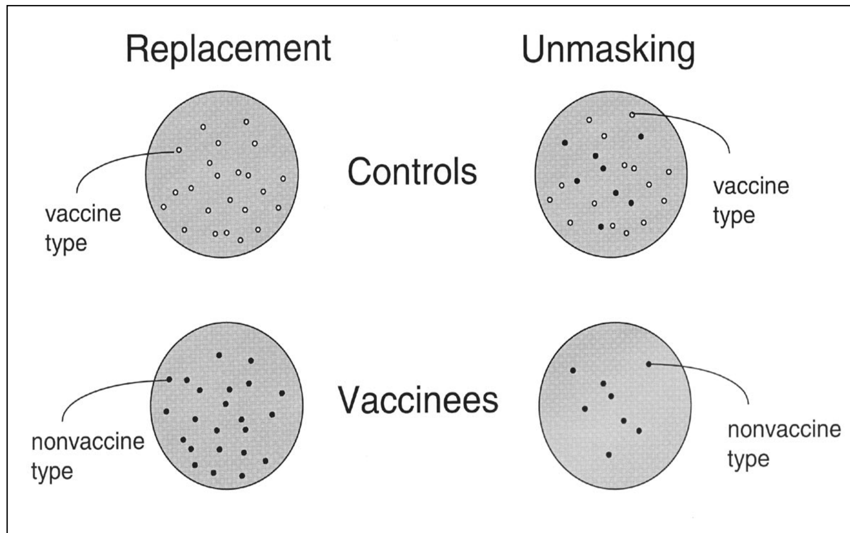

Figure 3. Two hypotheses explain the observation of higher rates of carriage of nonvaccine serotypes in vaccine recipients than in controls. Large circles represent plated samples from controls (top) andvaccine recipients (bottom). The left side shows true serotype replacement; here a control carries vaccine types (white colonies), while a vaccine recipient does not, and (possibly as a result of decreased competition) now carries only nonvaccine types (black colonies). The right side shows the unmasking phenomenon, which is an artifact of sampling. Here, both vaccinees and controls carry nonvaccine types, but because only one colony is sampled in each, the vaccinee does not appear to carry nonvaccine types.

vaccinated persons reflects true serotype replacement, unmasking, or a combination of these phenomena.

I have recently developed a statistical procedure to answer this question (M. Lipsitch, submitted for publication). The procedure attempts to detect serotype replacement by attempting to reject a null model that incorporates the effect of unmasking alone. In short, if the increase in nonvaccine type carriage in vaccinees, compared to controls, is greater than can be accounted for by this null model, then one concludes that additional factors, presumably serotype replacement, must be responsible for the observed increase. This technique has been applied to two datasets, one from South Africa (6) and one from Gambia (5). In both cases, the observed increase was greater than that expected from unmasking alone. In the South African case, the difference was statistically significant (p = 0.02), but it was not in the Gambian dataset (p = 0.085). However, the Gambian dataset was extremely small and some information was unavailable for this dataset that might have improved the power of the test. The test is simple to perform using the BUGS software (42,43) available free on the World Wide Web (http://www.mrc-bsu.cam.ac.uk/bugs/Welcome.html) and a program available from the author; thus, it may be readily applied to future datasets.

Limitations of Mathematical Models

The mathematical models described here, like all such models, involve a number of simplifications. In some cases, these simplifications are introduced to make the model more tractable and focus attention on fundamental processes of transmission and competition between serotypes. In other cases, the simplifications are necessary because much remains unknown about the biology—and especially the immunology—of carriage of these organisms. The assumptions of the model are discussed at greater length (33). One of these assumptions will be considered here in greater detail to highlight some areas where additional knowledge of the biology of pneumococcal-host interactions is most needed.

The model assumes that bacteria of different serotypes compete via direct interactions in the nasopharynx. These interactions may take the form of competition for resources, such as attachment sites or nutrients, or they may take the form of interference competition, in which a resident type produces substances toxic to other bacteria that may attempt to colonize the same host. Apart from the few studies cited above, little is known about either the intensity or the mechanisms of such inhibition. There are some epidemiologic data that indirectly indicate the existence of such competitive interactions. A study of military personnel in 1946 (27) used a very sensitive technique, mouse inoculation, to detect nasopharyngeal carriage of one or more pneumococcal serotypes. The numbers of persons carrying one, two, three, or four serotypes are given, and although the published data do not provide all of the information

necessary for formal statistical inference, the pattern suggests that interference between serotypes may have occurred.

The model does not take into account acquired immunity to carriage of these bacteria, or the possibility that carriage of one serotype may inhibit future carriage of another serotype, even after the first is no longer carried. It is unclear to what degree it is realistic to ignore acquired immunity to carriage. While carriage has been shown to induce a serum antibody response in at least one report (44), it is less clear whether such responses affect carriage at the nasopharyngeal mucosa. The success of conjugate vaccines in reducing carriage indicates that some antibody responses can affect carriage. However, it remains to be demonstrated whether such responses are induced by natural exposure through the respiratory route, whether natural exposure induces responses to other, more conserved antigens or only to the capsular antigen, and whether natural exposure induces long-lived immunologic memory. Preliminary results of mathematical models that incorporate naturally acquired immunity to carriage suggest that the expected effects of vaccination on the serotype composition of the population may be different from those expected under the models described here. Therefore, further research into the microbiology and immunology of the host-bacterial relationship in the nasopharynx will be critical to understanding and predicting the population-wide effects of conjugate vaccines.

Additional Considerations

The choice of serotypes for inclusion in conjugate vaccines has been different in different locations but has generally been designed to cover serotypes that are most often implicated in invasive disease. Often, these types coincide with serotypes showing the greatest levels of antibiotic resistance (45,46). As a result, conjugate vaccination has led to a reduction in the percentage of antibiotic-resistant pneumococci carried by vaccinees (4,6).

In principle, replacement could occur with bacteria that differ from the vaccine targets not only in serotype but in species. Indeed, one of the studies of bacterial antagonism in the nasopharynx concentrated on interactions between species rather than between serotypes of the same species (30). Furthermore, even if replacement is limited to members of the same species, the serotypes that increase may tend to cause a disease different from that caused by vaccine-type organisms (e.g., otitis rather than pneumonia or bacteremia). Therefore, as conjugate vaccines are used, changes in diseases attributable to organisms that colonize the nasopharynx should be monitored.

Finally, capsular polysaccharide is not the only possible target for vaccination. Several pneumococcal vaccines based on protein antigens are in various stages of testing (47). Because these protein antigens show considerably less variation among pneumococcal isolates, vaccines based on them should be less vulnerable to serotype replacement and may be useful as complements or alternatives to polysaccharide conjugate vaccines.

Conclusions

The occurrence of serotype replacement in three trials of pneumococcal conjugate vaccines confirms the validity of concerns expressed in anticipation of these trials. As the results of more clinical trials become available, it will become clearer how general this phenomenon is. Mathematical models are useful in suggesting ways to improve the design of these trials and the interpretation of their results.

The extent and importance of serotype replacement will depend on many locally variable factors, the prevalence of vaccine-type organisms before vaccination, and the level of vaccine coverage. This prediction underscores the need for continuing studies of vaccination in different communities and for at least some studies in which a substantial fraction of a community receives the vaccine. Furthermore, the epidemiologic findings of these studies should be the impetus for further research into the role of serotype and other factors in determining the variation in pneumococcal virulence, the nature of immune responses to organisms like the pneumococcus at the nasopharyngeal mucosal surface, and other questions in the biology of bacterial carriage.

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Iron Loading and Disease Surveillance

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Iron is an oxidant as well as a nutrient for invading microbial and neoplastic cells. Excessive iron in specific tissues and cells (iron loading) promotes development of infection, neoplasia, cardiomyopathy, arthropathy, and various endocrine and possibly neurodegenerative disorders. To contain and detoxify the metal, hosts have evolved an iron withholding defense system, but the system can be compromised by numerous factors. An array of behavioral, medical, and immunologic methods are in place or in development to strengthen iron withholding. Routine screening for iron loading could provide valuable information in epidemiologic, diagnostic, prophylactic, and therapeutic studies of emerging infectious diseases.

Excessive iron in specific tissues (iron loading) promotes infection, neoplasia, cardiomyopathy, arthropathy, and a profusion of endocrine and possibly neurodegenerative disorders (1-5). An array of behavioral, medical, and immunologic methods are being developed to decrease iron loading or its detrimental effects. Routine screening for iron loading in populations exposed to certain diseases can provide valuable epidemiologic, diagnostic, prophylactic, and therapeutic information.

Hazards of Iron Loading

Iron can contribute to disease development in several ways. Excessive amounts of the metal in specific tissues and cells can hinder the ability of proteins, such as transferrin and ferritin, to prevent accretion of free iron. Moreover, in infectious diseases, inflammatory diseases, and illnesses that involve ischemia and reperfusion, iron causes reactions that produce superoxide radicals (6). Nonprotein bound ferric ions are reduced by superoxide, and the ferrous product is reoxidized by peroxide to regenerate ferric ions and yield hydroxyl radicals, which attack all classes of biologic macromolecules. Hydroxyl radicals can depolymerize polysaccharides, cause DNA strand breaks, inactivate enzymes, and initiate lipid peroxidation (6).

Iron can also increase disease risk by functioning as a readily available essential nutrient for invading microbial and neoplastic cells. To survive and replicate in hosts, microbial pathogens must acquire host iron. Highly virulent strains possess exceptionally powerful mechanisms for obtaining host iron from healthy hosts (7). In persons whose tissues and cells contain excessive iron, pathogens can much more readily procure iron from molecules of transferrin that are elevated in iron saturation. In such cases, even microbial strains that are not ordinarily dangerous can cause illness. Markedly invasive neoplastic cell strains can glean host iron more easily than less malignant strains or normal host cells (3). Moreover, iron-loaded tissues are especially susceptible to growth of malignant cells (Table 1).

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Table 1. Iron loading in specific tissues and increased risk for disease

Tissue type	Disease
Alveolar macrophages	Pulmonary neoplasia and infection
Anterior pituitary	Gonadal and growth dysfunction
Aorta; carotid and coronary arteries	Atherosclerosis
Colorectal mucosa	Adenoma, carcinoma
Heart	Arrhythmia, cardiomyopathy
Infant intestine	Botulism, salmonellosis, sudden death
Joints	Arthropathy
Liver	Viral hepatitis, cirrhosis, carcinoma
Macrophages	Intracellular infections
Pancreas	Acinar and beta cell necrosis, carcinoma
Plasma and lymph	Extracellular infections
Skeletal system	Osteoporosis
Skin	Leprosy, melanoma
Soft tissue	Sarcoma
Substantia nigra	Parkinson's disease

How Microbes Acquire Iron: A Determinant of Host Range and of Tissue Localization

The number of infectious disease agents whose virulence is enhanced by iron continues to increase (Table 2). To obtain host iron, successful pathogens use one or more of four strategies: binding of ferrated siderophilins with extraction of iron at the cell surface; erythrocyte lysis, digestion of hemoglobin, and heme assimilation; use of siderophores that withdraw iron from transferrin; and procurement of host intracellular iron.

Microbial strains that use siderophilin binding often have a very narrow host range (7). Bacterial receptors recognize siderophilins generally from a single or closely related host species. Strains of *Haemophilus somnus*, for example, form receptors for bovine but not for human transferrin; these bacteria are virulent for cattle but not for humans (9). The human pathogen, *Neisseria meningitidis*, can bind ferrated transferrins from humans and such hominids as chimpanzees, gorillas, and orangutans, but not from monkeys or nonprimate mammals (10,11). *Actinobacillus pleuropneumoniae* synthesizes a swine-specific transferrin receptor and causes pneumonia only in hogs (12).

Each of the above three pathogens, as well as other organisms that use siderophilin binding, can often obtain iron from heme. *Helicobacter pylori*, for instance, first obtains iron from human ferrated lactoferrin in the gastric lumen. Then, as it migrates into intercellular junctions of epithelial cells in the gastric wall, its sole source of iron is heme. This pathogen binds neither bovine ferrated lactoferrin nor human, bovine, or equine ferrated transferrin (13).

However, not every pathogen that uses siderophilin binding has a narrow host range. For example, *Staphylococcus aureus* can be virulent for a variety of mammalian species. Strains of this organism can bind human, rat, and rabbit transferrins and, much less efficiently, bovine, porcine, and avian transferrins (14). Moreover, isolates of *S. aureus* also may produce siderophores (15,16). These small molecules can withdraw iron from transferrins synthesized by a variety of host species. The siderophore, staphyloferrin A, removes iron from both human and porcine transferrin; thus, the metal can be available to invading cells in humans and in hogs. Erythrocyte lysis, digestion of hemoglobin, and heme assimilation are available to strains of *S. aureus*. Bacterial hemolysins generally are active against erythrocytes from several, although not from all, potential host species.

Virulent streptococci are examples of bacteria that neither bind siderophilins nor produce siderophores yet proficiently invade and replicate in many tissues in diverse host species. The cellulytic activities of these pathogens enable them to access such intracellular sources of host iron as hemoglobin, myoglobin, catalase, and ferritin (17).

The remarkable versatility for host species shown by *Listeria monocytogenes* illustrates the adeptness of this organism in procuring iron. Although mainly a saprophyte that lives in the plant-soil environment, *L. monocytogenes* can be acquired by humans and other mammals through ingestion of undercooked tissue of other mammals, birds, fish, and Crustacea, as well as from raw vegetables. Unable to bind siderophilins or form siderophores, *L. monocytogenes* obtains

Table 2. Microbial genera with strains whose growth in body fluids, cells, tissues, and intact vertebrate hosts is stimulated by excess iron (8)

Fungi	Protozoa	Gram-positive and acid-fast bacteria	Gram-negative bacteria	
Candida	*Entamoeba*	*Bacillus*	*Acinetobacter*	*Klebsiella*
Cryptococcus	*Leishmania*	*Clostridium*	*Aeromonas*	*Legionella*
Histoplasma	*Naegleria*	*Corynebacterium*	*Alcaligenes*	*Moraxella*
Paracoccidioides	*Plasmodium*	*Erysipelothrix*	*Campylobacter*	*Neisseria*
Pneumocystis	*Toxoplasma*	*Listeria*	*Capnocytophaga*	*Pasteurella*
Pythium	*Trypanosoma*	*Mycobacterium*	*Chlamydia*	*Proteus*
Rhizopus		*Staphylococcus*	*Ehrlichia*	*Pseudomonas*
Trichosporon		*Streptococcus*	*Enterobacter*	*Salmonella*
			Escherichia	*Shigella*

iron by using either exogenous siderophores of other microorganisms or natural catechols, such as dopamine and norepinephrine, in host tissues. The pathogen expresses a cell surface ferric reductase that recognizes the siderophoric chelated iron site; the metal is then reduced and assimilated (18). Furthermore, in contrast to saprophytic strains, systemic pathogenic strains of *L. monocytogenes* are hemolytic.

To grow within host cells, pathogens apparently are not required to synthesize siderophilin binding sites or form siderophores. For instance, unlike the wild type, siderophore-minus mutants of *Salmonella* Typhimurium cannot grow in extracellular compartments of the host. However, both the wild and mutant strains replicate within host cells (19). Possible sources of intracellular iron are heme, iron released from transferrin at pH 5.5-6, and ferritin.

For at least two pathogens, *Francisella tularensis* and *Legionella pneumophila*, the host intracellular niche is obligatory. Like the mutant strain of *S.* Typhimurium, these organisms are unable to access iron in extracellular fluids and tissues. Culturing these bacteria in laboratory media requires markedly elevated concentrations of iron (20,21).

In host intracellular niches, growth of microbial pathogens is stimulated by elevation and depressed by decrease of iron. Indeed, at least one bacterial pathogen, *Ehrlichia chaffeensis*, induces elevation of iron in its host cells; intracellular inclusions of the organism cause the host cell to upregulate expression of the transferrin receptor mRNA (22).

Iron Withholding Defense System

Hosts use several mechanisms (Table 3) to withhold iron from invading microbial and neoplastic cells: stationing of potent iron binding proteins at sites of impending microbial invasion; lowering iron levels in body fluids, diseased tissues, and invaded cells during invasion; and synthesizing immunoglobulins to the iron acquisition antigens of microbes.

High concentrations of iron not only benefit invading cells, they may also mediate antimicrobial activities of defense cells. In in vitro studies, 150 µM iron augmented macrophage killing of *Brucella abortus* (24) and, without altering phagocytosis, 250 µM iron enhanced anti-Candida activity of microglia (25). In the latter system, the metal suppressed synthesis of nitric oxide but not of tumor necrosis factor A. By generating oxidant-sensitive mediators, iron may focus influx of neutrophils to sites of infection (26). Iron loading of staphylococci increased their killing by peroxide, macrophages,

Table 3. The iron withholding defense system (1,8)

Constitutive components
Siderophilins
Transferrin in plasma, lymph, cerebrospinal fluid
Lactoferrin in secretions of lachrymal and mammary glands and of respiratory, gastrointestinal, and genital tracts
Ferritin within host cells
Processes induced at time of invasion
Suppression of assimilation of 80% of dietary iron[a]
Suppression of iron efflux from macrophages that have digested effete erythrocytes to result in 70% reduction in plasma iron[a]
Increased synthesis of ferritin to sequester withheld iron[a]
Release of neutrophils from bone marrow into circulation and then into site of infection[a]
Release of apolactoferrin from neutrophil granules followed by binding of iron in septic sites
Macrophage scavenging of ferrated lactoferrin in areas of sepsis and of tumor cell clusters
Hepatic release of haptoglobin and hemopexin (to bind extravasated hemoglobin and hemin, respectively)
Synthesis of nitric oxide (from L-arginine) by macrophages to disrupt iron metabolism of invaders[b]
Suppression of growth of microbial cells within macrophages via downshift of expression of transferrin receptors and enhanced synthesis of Nrampl (23) by the host cells[b]
Induction in B lymphocytes of synthesis of immunoglobulins to iron-repressible cell surface proteins that bind either heme, ferrated siderophilins, or ferrated siderophores

[a]Activated by interleukin-1 or -6 or by tumor necrosis factor-α.
[b]Activated by interferon-γ.

and neutrophil-derived cytoplasts but not by neutrophils (27). Certain conditions can impair iron withholding (Table 4); numerous studies have presented evidence that risk for infection or neoplasia is increased significantly in persons with these conditions.

Table 4. Conditions that can compromise iron withholding (1,3)

- Excessive intake of iron through intestinal absorption
  - Behavioral and nutritional factors
    - Accidental ingestion of iron tablets
    - Adulteration of processed foods with inorganic iron or blood
    - Excessive consumption of red meats (heme iron)
    - Excessive intake of alcohol (HCl secretion enhanced)
    - Folic acid deficiency
    - Ingestion of ascorbic acid with inorganic iron
    - Use of iron cookware
  - Genetic and physiological factors
    - African siderosis
    - Asplenia (mechanism unknown)
    - Pancreatic deficiency of bicarbonate ions
    - Porphyria cutanea tarda
    - Regulatory defect in mucosal cells in hemochromatosis
    - Thalassemia, sicklemia, other hemoglobinopathies
- Parenteral iron
  - Intramuscular and intravenous iron saccharate injections in excess
  - Multiple transfusions of whole blood or erythrocytes in excess
- Inhaled iron
  - Exposure to amosite, crocidolite, or tremolite asbestos
  - Exposure to urban air particulates
  - Mining iron ore, welding, grinding steel
  - Painting with iron oxide powder
  - Tobacco smoking (1-2 µg iron inhaled per cigarette pack)
- Release of body iron from compartments into plasma
  - Efflux of erythrocyte iron in hemolytic diseases
  - Efflux of hepatocyte iron in hepatitis
- Deficit in iron withholding
  - Transferrin
  - Decreased synthesis
    - Congenital defect
    - Lack of dietary amino acids in kwashiorkor or in jejunoileal bypass
    - Decreased activity in acidosis
  - Lactoferrin
    - Neutropenia
    - Substitution of bovine milk or milk formula for human milk in nursling nutrition
  - Haptoglobin
    - Decreased synthesis in persons with haplotype 2-2 (28)

Detection of Iron Loading

Screening of large populations for iron loading can be accomplished with inexpensive, noninvasive methods. A useful indicator of iron loading is marked elevation of serum ferritin (sFt). However, sole reliance on this measurement can be misleading because sFt increases moderately during inflammatory episodes. Accordingly, concurrent determination of the percentage of iron saturation of serum transferrin (%TS) provides useful information (29). In iron loaded persons, hyperferritinemia generally is accompanied by an elevation in %TS. In contrast, in patients with an inflammatory process, hyperferritinemia generally is accompanied by a reduction in %TS.

Iron loading is associated also with moderate depression of a third variable, serum transferrin receptor (sTfR). The ratio of sTfR/sFt, apparently independent of inflammation, is significantly reduced in persons with high levels of iron (5).

Strengthening the Iron Withholding Defense

A considerable array of behavioral, medical, and immunologic methods are in place or in development for strengthening iron withholding (Table 5) (3). Additional precautions are indicated for persons who are known to be (or have a tendency to become) iron loaded. For example, persons with elevated iron due to either hemochromatosis or alcoholism are cautioned to avoid eating raw oysters, which may contain *Vibrio vulnificus* (30). Another pathogen that likewise causes severe systemic infection in hosts with elevated iron is *Capnocytophaga canimorsis*. Accordingly, persons who have hemochromatosis, alcoholism, or asplenia are advised to receive prompt antibiotic therapy if they are exposed to a dog bite (31).

De-ironing by phlebotomy is effective in lowering risk for cardiovascular diseases (32,33) and various neoplasms (34), as well as in therapy for hepatitis C (35). Interfering with iron metabolism by administering gallium can be useful in suppressing growth of lymphoma and bladder cancer cells (36). The antineoplastic action of monoclonal antibodies against ferrated transferrin receptors has been examined (37).

Table 5. Methods of strengthening the iron withholding defense system

Reduction of excessive intake of ingested iron
Decreased consumption of red meats (heme iron)
Avoidance of processed foods that have been adulterated with inorganic iron or with blood
Decreased consumption of alcohol and ascorbic acid
Elimination of iron supplements unless an iron deficiency has been correctly diagnosed
Reduction of excessive intake of parenteral iron
Inject iron saccharates only if unequivocally justified
Transfuse blood or erythrocytes only if unequivocally justified
Substitute erythropoietin (+ minimal amount of iron) for whole blood transfusions when possible
Reduction of excessive inhalation of iron
Eliminate use of tobacco
Use iron-free chrysotile in place of iron-loaded amosite, crocidolite, tremolite varieties of asbestos
Use mask to avoid inhalation of urban air particulates
Use mask and protective clothing when mining or cutting ferriferous substances
Reduction of iron burden by regular depletion of whole blood or erythrocytes
Avoidance of premature hysterectomy
Routine ingestion of aspirin
Regular donations of whole blood or erythrocytes
Vigorous exercise
Increased use of iron chelators
Use human milk (high in lactoferrin, low in iron) rather than milk formula (lacking in lactoferrin, high in iron) in nursling nutrition
Use tea (iron-binding tannins) and bran (iron-binding phytic acid)
Continue research and development (R&D) of potential iron chelator drugs (e.g., recombinant human lactoferrin; hydroxpyridones; pyridoxal isonicotinoyl hydrazones)
Initiation of prompt therapy of chronic infections and neoplastic diseases to forestall saturation of iron withholding defense system
Continued R&D of cytokines such as interferon γ that induce cellular iron withholding
Continued R&D of passive and active methods of immunization against surface receptor proteins used by microbial and neoplastic cells to obtain iron

Combinations of the iron chelator, deferoxamine, with gallium or with antibodies against ferrated transferrin receptors increase effectiveness against tumor cells.

The natural iron scavenger, lactoferrin, has been shown to remove free iron from synovial fluid aspirated from joints of rheumatoid arthritic patients (38). Recombinant human lactoferrin, which is indistinguishable from native breast milk lactoferrin with respect to its iron binding properties, is now available (39) and could become a very useful addition to our array of de-ironing pharmaceutical products.

A recently discovered integral membrane phosphoglycoprotein, Nrampl, is expressed exclusively in macrophages and is localized to phagolysosomes. The protein suppresses replication of intramacrophage microbial invaders apparently by altering iron availability (23). A second protein, Nramp2, is involved in enhancement of intestinal iron absorption (40). Future research might develop useful medical procedures for modulation of the actions of these proteins.

Potential vaccines that incorporate iron acquisition antigens of pathogens in the families *Neisseriaceae* and *Pasteurellaceae* are being developed by several research groups. For example, in *Moraxella catarrhalis*, the recombinant transferrin binding protein B (TbpB) has been shown to elicit bactericidal antibodies (41) In *N. meningitidis*, antisera to TbpA and TbpB were bactericidal for both homologous and heterologous strains (42,43). Because the antigenic proteins function at the cell surfaces of the pathogens, the receptors are potentially ideal vaccine candidates. For synthesis of the receptors, the organisms must be cultured in iron-restricted media.

Perspectives and Conclusions

There is growing awareness that transmissible agents are involved in diseases not earlier suspected of being infectious (44-46). A recent

review contains a list of 34 degenerative, inflammatory, and neoplastic diseases associated in various ways with specific infectious agents (44). Other chronic inflammatory diseases, such as sarcoidosis, inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, Wegener granulomatosis, diabetes mellitus, primary biliary cirrhosis, tropical sprue, and Kawasaki disease may also have infectious etiologies (45). Excessive iron is correlated with synovial damage in rheumatoid arthritis (47) and with impaired glucose metabolism in diabetes (48). The association of *Chlamydia pneumoniae* (49) and excessive iron (5) with cardiovascular disease is well established. Growth of this pathogen is strongly suppressed by iron restriction (50).

Proving the role of infection in chronic inflammatory diseases and cancer presents challenges (46). The means by which pathogens suppress, subvert, or evade host defenses to establish chronic or latent infection have received little attention. However, the association and causal role of infectious agents in chronic inflammatory diseases and cancer have major implications for public health, treatment, and prevention (44-46).

Iron loading is a risk factor in these illnesses, as well as in classic infectious diseases. Because the prevalence of iron loading in various populations can be remarkably high, routine screening of iron values in host populations could provide valuable information in epidemiologic, diagnostic, prophylactic, and therapeutic studies of emerging infectious diseases.

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Human Herpesvirus 6: An Emerging Pathogen

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Infections with human herpesvirus 6 (HHV-6), a ß-herpesvirus of which two variant groups (A and B) are recognized, is very common, approaching 100% in seroprevalence. Primary infection with HHV-6B causes roseola infantum or exanthem subitum, a common childhood disease that resolves spontaneously. After primary infection, the virus replicates in the salivary glands and is shed in saliva, the recognized route of transmission for variant B strains; it remains latent in lymphocytes and monocytes and persists at low levels in cells and tissues. Not usually associated with disease in the immunocompetent, HHV-6 infection is a major cause of opportunistic viral infections in the immunosuppressed, typically AIDS patients and transplant recipients, in whom HHV-6 infection/reactivation may culminate in rejection of transplanted organs and death. Other opportunistic viruses, human cytomegalovirus and HHV-7, also infect or reactivate in persons at risk. Another disease whose pathogenesis may be correlated with HHV-6 is multiple sclerosis. Data in favor of and against the correlation are discussed.

The Discovery of Human Herpesvirus 6 (HHV-6)

Initially designated HBLV, for human B-lymphotropic virus, HHV-6 was isolated fortuitously in 1986 from interleukin 2-stimulated peripheral blood mononuclear cells (PBMCs) of patients with AIDS or lymphoproliferative disorders (1). The PBMC cultures exhibited an unusual cytopathic effect characterized by enlarged balloonlike cells. The causative agent was identified as a herpesvirus by electron microscopy and lack of cross-hybridization to a number of human herpesviruses (2). The GS strain is the prototype of the first isolates. Two additional isolates of lymphotropic human herpesviruses, U1102 and Gambian, genetically similar to HBLV, were obtained 1 year later from PBMCs of African AIDS patients. All of the isolates could grow in T cells (CEM, H9, Jurkat), in monocytes (HL60, U937), in glial cells (HED), as well as in B-cell lines (Raji, RAMOS, L4, WHPT) (3,4). A new variant, Z29, subsequently shown to differ in restriction endonuclease pattern from GS-like strains, was isolated from PBMCs of patients with AIDS (5). The cells supporting virus growth were characterized as CD4+ T lymphocytes (6). The designation HHV-6 was proposed 1 year after discovery of the first isolate to comply with the rules established by the International Committee on Taxonomy of Viruses (7).

More than 100 additional HHV-6 strains have been isolated from PBMCs of children with subitum or febrile syndromes (8), from cell-free saliva of healthy or HIV-infected patients (9,10), from PBMCs of patients with chronic fatigue syndrome (CFS) (11), and from PBMCs of healthy adults—these PBMCs were cultivated for human herpesvirus 7 (HHV-7) isolation (12).

The Virus

HHV-6 and HHV-7 belong to the *Roseolovirus* genus of the ß-herpesvirus subfamily; HHV-6 species are divided into two variants: HHV-6A and HHV-6B. The virion particle is 160 nm to 200 nm and has the morphologic features typical of herpes virion particles (a central core containing the viral DNA, a 90-nm to 110-nm capsid, and a tegument layer surrounded by a membrane structure) (13). We summarize briefly key features.

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HHV-6A genomes are 159 kbp to 170 kbp long. As sequenced, the genome of U1102 strain is 159 kbp long (14); the HHV-6B genome has been sequenced only partially. Seven gene blocks in the central region (I-VII) designated as herpesvirus core genes are common to all Herpesviridae. Another block, spanning open reading frames (ORFs) U2 to U14, contains genes specific to ß-herpesviruses. A further region, encompassing ORFs U15-U25, contains genes specific to *Roseolovirus* genus. Three genes (U22, U83, U94) are specific to HHV-6 and absent from HHV-7 (Figure 1). The closest homology and similarity in genome organization is to HHV-7 and next to human cytomegalovirus (HCMV). Amino acid similarity to HHV-7 is 46.6% to 84.9% and to HCMV 41.0% to 75.8% (14,16). The HHV-6 genome is composed of a unique sequence (85% to 90% of the genome) bracketed by direct repeats (10% to 15% of the genome) that contain the cleavage and packaging sequences *pac*-1 and *pac*-2 and a single origin of replication (OriLyt) located at 70 kbp of the genome. The number of predicted ORFs, 102 or 85, varies depending on the values used to define an ORF and was attributed mainly on the basis of the similarity with HCMV (14) or HHV-7 (17) counterparts. Few gene products have been characterized so far. They include the immediate-early gene IE-A, which together with IE-B constitutes the IE locus, a highly spliced region with an arrangement similar to that of HCMV (18); the U3 gene, a homolog of the HCMV U_L24 gene, with transactivating activity (19); the origin binding protein (20), the U53 protease (21); and p100, also designated p101, highly immunogenic, and most probably a constituent of the tegument (22,23). In addition, HHV-6 (but not HHV-7) carries a homolog of the adenoassociated type 2 parvovirus *rep* gene (24), which is transcribed in latently infected cells (25). Recently, the U12 protein was recognized as a ß-chemokine receptor (26). A major focus has been in the glycoprotein field. Five glycoproteins were identified: gB (U39, gp116) (27-30), gH (U48, gp100) (31), and gL (U82, gp80), which form at least a heterodimer, gM (U72), and gp82-105 (U100) (29,30,32,33). gB and gH/gL were shown to be virion constituents, and antibody to gH can neutralize virion infectivity and syncytia formation, suggesting a role of gH in virus entry and in virus-induced cell fusion (31). The HHV-6 genome sequence predicts a locus of glycoproteins U20-U24 and U85 that are specific to the *Roseolovirus* genus (14), but the proteins have not yet been identified. U20 and U85 have a predicted immunoglobulin structure.

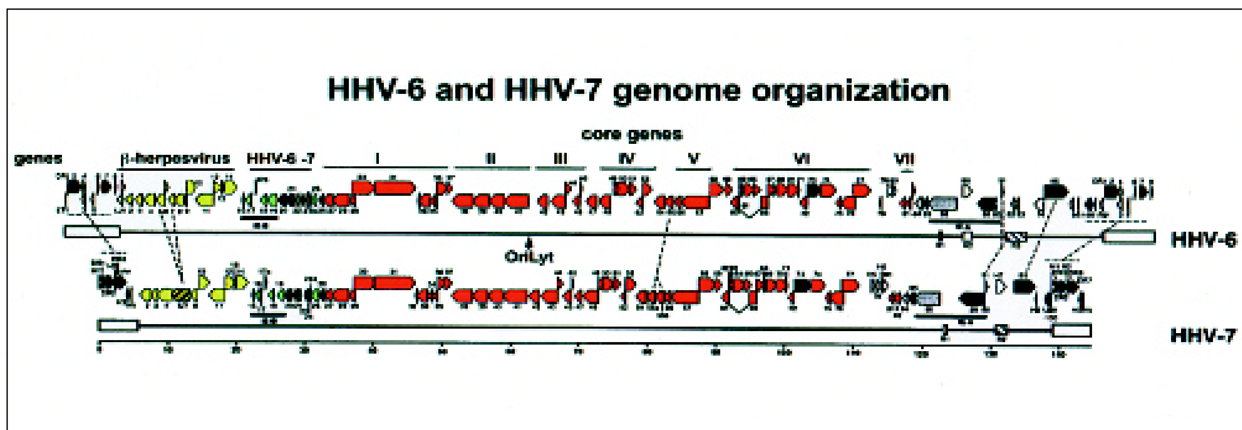

Figure 1. Schematic representation of HHV-6 and HHV-7 genomes. The genomes are colinear. Homologies are 46.6% to 84.9%. Red blocks represent the herpesvirus core genes, numbered from I to VII. Yellow blocks represent ß-herpesvirus subfamily-specific genes (from U2 to U14). Green blocks indicate genes present only in the *Roseolovirus* genus, i.e., in HHV-6 and HHV-7. Only three ORFs (U22, U83 and U94) are present in HHV-6 and absent from HHV-7 (modified from [15]).

Variant A and Variant B HHV-6 Strains

Frenkel and co-workers (34), Ablashi et al. (35), and Aubin et al. (36) were the first to

discover that HHV-6 isolates display genetic and phenotypic variations. All the strains derived so far segregate into two groups, variant A and variant B, whose genome organization appears to be overlapping. Viruses belonging to the two variants differ with respect to several properties. Differences in restriction endonuclease cleavage sites are scattered throughout the entire genomes. Extent of homologies at nucleotide level varies from 99% to 95% for the most conserved genes located in the center of the genome to approximately 75% for the most divergent portions, located in the immediate-early region. Major differences in biologic properties concern the in vitro cell tropism, regulation of transcription and splicing patterns, reactivity to some MAbs directed to variant-specific epitopes (29,34,35). Typically, variant A viruses replicate in HSB-2 cells, whereas the variant B viruses grow in the less differentiated Molt3 T-cell lines. Variant B viruses grow to higher yields than variant A viruses in primary human fetal astrocytes and require IL-2- and phytohemagglutinin-activated PBMCs. Differences between the two variants affect the regulation of transcription of some ORFs of the immediate early region-B and-A (U16, U17, U91) and the splicing pattern of ORFs U18-U20 (37). The differences relative to infection in humans (epidemiology, correlation with pathologic features, tissue tropism) are detailed below and in the table.

All strains fall into one or the other variant group. There is no evidence of recombination or a genetic gradient, which suggests that in vivo the two groups of viruses occupy different ecologic niches. Any isolate characterized for more than one marker has been unambiguously assigned to one or the other variant group. The designation of the two groups as variants has been highly debated and controversial (38). A key question is whether the two variants fulfill the criteria defined by the International Committee on Taxonomy of Viruses for classification as different species (13). In our opinion, the information summarized above indicates that the two variant groups may be different species; therefore, the issue of nomenclature should be reconsidered.

Table. Epidemiology and distribution of human herpesvirus (HHV-6) variants

	Variant A	Variant B
Associated pathologic conditions		
Exanthem subitum, febrile syndromes[b]	–[a]	++++
Multiple sclerosis	++	++
Lymphomas and neoplasies	++	++
Reactivation in transplants	++	++
Reactivation in AIDS	++	++
Tissue distribution		
Peripheral blood mononuclear cells	+	+++
Salivary glands	–	++++
Skin	++	++
Brain	++	++
Lymph nodes	–	++++
Other tissues	–	++++
Serum from healthy persons	–	–
Serum from exanthem subitum patients	–	++++
Serum from other patients[c]	+++	+
Saliva	–	++++
Cerebrospinal fluid	+++	+

[a]Different degrees of HHV-6 positivity.
[b]Exception, Zambian children, 44% variant A.
[c]Patients with AIDS, chronic fatigue syndrome, and lymphomas.

Natural History of HHV-6 Infection

Infection with HHV-6 is very common, approaching 100% in seroprevalence. Exceptions, if confirmed, are represented by countries (e.g., Morocco) where seroprevalence is much lower (20%) (39). Antibody titers are high in newborn children, drop at 3 to 9 months after birth, rise again briefly thereafter, and remain elevated until the age of 60 or older. This pattern indicates that newborns carry maternal antibodies and primary infection occurs in the first 3 years of life, most frequently the first year. Transplacental infections are very infrequent but may contribute to HHV-6 seropositivity in newborns (40).

Three stages can be recognized in the natural history of HHV-6 infection (Figure 2). The first is represented by acute primary infection in infants. The second occurs in healthy children and adults; the virus replicates in the salivary glands and is secreted in saliva (for HHV-6B) without inducing any obvious pathology, remains latent at least in lymphocytes and monocytes, and persists in various tissues, possibly with a low-level replication. The third stage occurs infrequently, typically in immunocompromised persons, and is linked to reactivation of virus from latency or reinfection.

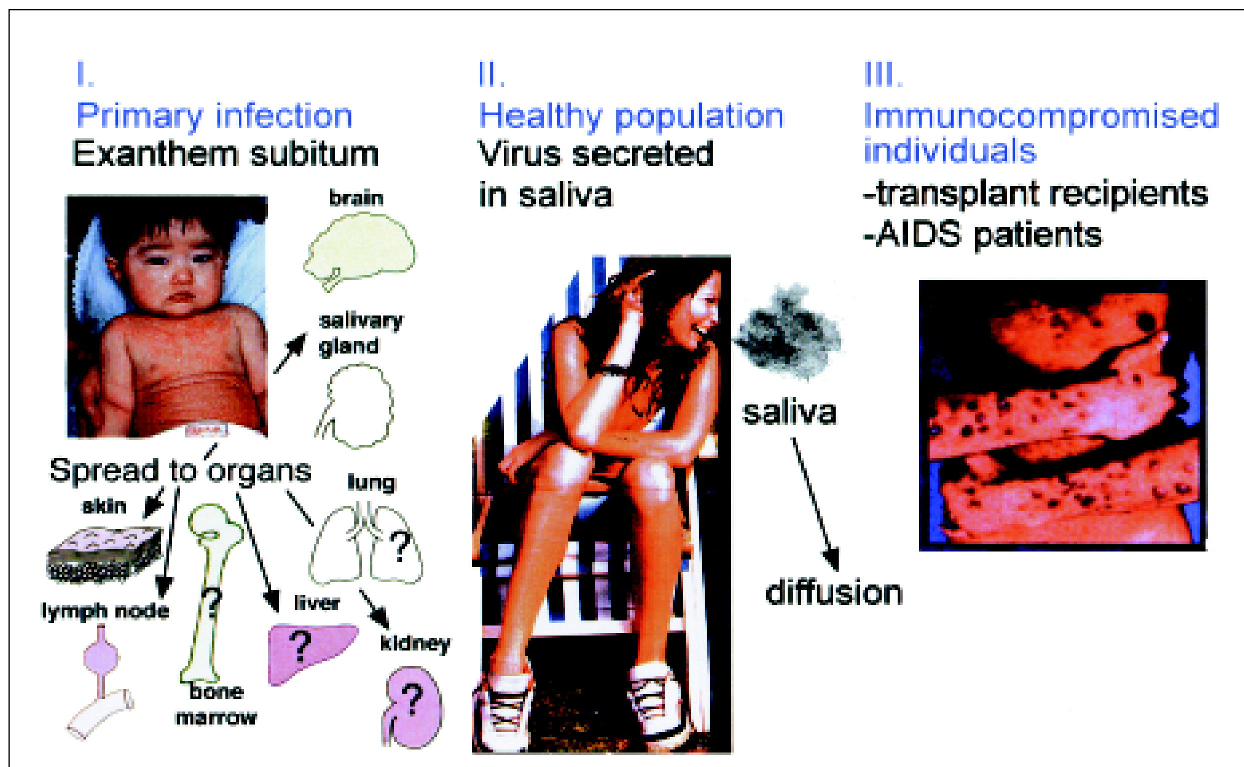

Figure 2. Stages of the natural history of HHV-6 infection: I. Primary infection occurs in infants, may result in exanthem subitum (rash on the child's chest), and spreads to organs. Question marks denote sites where HHV-6 spread is likely but not proven. II. In healthy infants and adults, HHV-6 is present in a latent or persistent form in lymph nodes and is produced asymptomatically in salivary glands and shed in saliva, the most probable route of transmission. III. HHV-6 infection/reactivation occurs in persons undergoing therapeutic immunosuppression after organ transplant or in AIDS patients.

Other pathologic conditions, mainly multiple sclerosis, tumors, and CFS have been linked to HHV-6.

Primary Infection

The unequivocal demonstration that primary infection with HHV-6B causes roseola infantum was provided by Yamanishi et al. (8), who investigated the correlation between seroconversion to HHV-6B and childhood infectious diseases and found that seroconversion occurs concomitantly with roseola infantum, also designated exanthem subitum or sixth disease, a common, mild, acute febrile disease of infants. Fever lasts for a few days and is sometimes followed by a maculopapular rash that resolves spontaneously. Primary infection may be asymptomatic or may cause clinical manifestations other than classic exanthem subitum. In four studies, children admitted to emergency clinics with febrile illnesses were HHV-6-positive in approximately 10% to 15% of cases and in one study in approximately 45% of cases, as determined by viral isolation, seroconversion, or detection of viral DNA sequences in PBMCs. Other than rash, symptoms included otitis, gastrointestinal or respiratory distress, and seizures (41-44). Complications of primary HHV-6 infections are uncommon and rarely fatal; they were described mainly as case reports and include invasion of the central nervous system (CNS) with seizures, hyperpyrexia, vomiting, diarrhea, cough, emophagocytic syndrome, fulminant hepatitis, disseminated infection, and hepatosplenomegaly. These complications suggest that the virus may spread to a number of organs, which may represent potential sites of virus persistence or latency and (subsequently) reactivation. For example, seizures and other CNS complications are clear indications of invasion of this organ and correlate well with neurotropism of HHV-6.

HHV-6 primary infection accounts for 10% to 45% of cases in children admitted to emergency clinics with febrile illness and 1% of cases in hospitalized children (42).

HHV-6B is not the only causative agent of exanthem subitum. Occasionally, HHV-7 may also cause fever with or without rash. Primary infection with HHV-7 occurs at a somewhat later age than with HHV-6B. Initially, it was proposed that pathologic manifestations seen during primary HHV-7 infection were the consequence of HHV-6 reactivation by HHV-7. Evidence that HHV-7 by itself causes an exanthematic disease, although less frequently than HHV-6B, rests on the finding that children with exanthem subitum seroconvert to HHV-7 but remain HHV-6B-negative (45,46).

Virus replicated in the salivary glands and secreted in saliva is the epidemiologically proven source of transmission. Other routes of transmission have been suggested but remain to be proven. HHV-6B DNA was recovered from cervical tissues and secretions (47-49), but children born to mothers with positive cervical swabs did not acquire the infection. Intrauterine transmission was suggested by polymerase chain reaction (PCR) positivity of uncultured cord blood mononuclear cells (CBMCs) in 1.6% of the cases and by a case of abortion of an HHV-6-positive fetus (40). Transmission through breastfeeding is also doubtful since HHV-6 DNA, unlike HHV-7, is not found in breast milk (50). Integration of the HHV-6 genome in lymphoblasts from a leukemic patient and his offspring raised the possibility of genetic transmission. As vertical transmission was not observed in other cases of genome integration, the presence of HHV-6 DNA in offspring was alternatively interpreted as a tendency of HHV-6 to integrate at specific chromosomal loci (51,52).

With the exception of a strong association of HHV-6A with febrile syndromes in Zambian children (43), which could reflect an endemic variant A hot spot, HHV-6A has rarely been isolated or detected in children with primary HHV-6 infection (53,54). The age at which primary HHV-6A infection occurs and the diseases clearly linked to it have not been determined.

HHV-6 in Healthy Persons

The second stage of HHV-6 infection occurs in healthy children and adults, in whom the virus actively replicates in the salivary glands, is latent in at least lymphocytes and monocytes, and persists in various tissues. Replication in salivary glands—observed for HHV-6B but not HHV-6A (9,10,47)—accounts for the route of transmission and for the high frequency of detection and isolation of virus in saliva. Lymphocytes, and probably monocytes, represent two known sites of latency, as the virus can be reactivated from PBMCs and adherent monocytes upon cultivation (55), and viral DNA sequences are detected in PBMCs of as much as 90% of the population. Additional sites of latency likely exist since the virus or viral sequences can be readily detected in a number of tissues. A form of latent infection is represented by integration of the HHV-6 genome in the host chromosomes (51,52). Persistence of HHV-6 in cells and tissues is discussed in the section In Vivo Tropism.

A missing link in our understanding of the natural history of HHV-6 infection is the source of the virus that spreads to organs. Monocytes have a short half-life; they may be vehicles of virus spread to organs, but they themselves need to be infected. A possible source may be virus produced in the salivary glands. In one case, early bone marrow progenitor cells were found to be latently infected in vivo (56), which raises the possibility that they may represent a site of latency, and by corollary, upon viral reactivation from latency, an alternative source by which virus spreads to tissues.

In immunocompetent adults, infection or reactivation of HHV-6 at sites other than the salivary glands is rare. Occasionally, infection results in lymphoadenopathy, fulminant hepatitis, mononucleosislike syndrome, or generalized infection.

HHV-6 in the Immunosuppressed

The third stage of HHV-6 infection, which occurs in the immunosuppressed, is responsible for the most serious clinical manifestations associated with HHV-6 infection or reactivation. Persons at risk are recipients of bone marrow, kidney, and liver transplants, in whom immunosuppression is induced for therapeutic reasons. In these patients, HHV-6 infection or reactivation may result in bone marrow suppression, pneumonitis, encephalitis, encephalopathy, hepatitis, fever, and skin rash or may complicate engraftment of the transplanted organ and culminate in rejection and death. As

the number of persons undergoing organ transplantation and, consequently, subjected to therapeutic immunosuppression increases, the number of persons at risk is increasing. Assessment of the contribution of HHV-6 to posttransplant complications is made more difficult by the presence of other opportunistic viruses and by the scarcity of thorough studies on all the viruses present in these organs. Thus, most of the reports on the presence of HHV-6 did not deal with the fact that HCMV reactivation is frequent in transplant recipients (particularly kidney) and may occur together with HHV-6 reactivation. When investigated in detail, a synergistic effect of HHV-6 and HCMV was apparent in renal transplant recipients, and the simultaneous detection of both HHV-6 and HCMV DNAs in urine or serum or of immunoglobulin (Ig) M antibodies was the strongest predictor of viral disease and of severity of disease (57,58). HHV-7 can also reactivate in transplant recipients (59), again alone and in association with HHV-6 or HCMV. Each of these viruses is a pathogen in its own right, and in combination with the other, may produce disease far more serious in outcome and clinical manifestations than it would alone. In many studies, no effort was made to identify the HHV-6 variant. When the variants were characterized, a rather heterogeneous pattern emerged. In PBMCs, brain and lungs variant B strains were predominant (60-62), whereas in spinal fluid and serum, variant A strains were prevalent (63,64). In approximately 30% of bone marrow transplant recipients in whom pneumonitis developed, both variants were simultaneously detected (62), an otherwise rare occurrence.

AIDS patients are the second group of immunocompromised persons at risk for HHV-6 and HCMV-related opportunistic viral infections. The overall incidence of these infections has decreased substantially after the introduction of highly active antiretroviral therapy. HHV-6 infection/reactivation in AIDS patients results in an increase in HHV-6 load both in lymph nodes and generalized, in viremia, disseminated infection in many organs, active CNS infection, pneumonitis, and retinitis and may contribute as a cause of death (65-67). These findings lead to the proposal that HHV-6 acts as a cofactor in the progression of AIDS and in the switch of HIV from the latent to the replicative state (68). Although a significant increase in HHV-6 viral load was not observed in PBMCs of HIV-seropositive persons (68,69), HHV-6 and HIV could interact in lymph nodes. The possibility that HHV-6 acts as a cofactor in AIDS progression boosted intense research on mutual interactions between HHV-6 and HIV in cell cultures and cell-free systems. In addition to coinfection, observed in vivo and in vitro, HHV-6 promotes HIV replication through upregulation of cytokines (e.g., TNF-α and IL-1ß) and through transactivation of the long terminal repeat by IE-A and IE-B (68). The possibility that in vivo HHV-6 infection may lead to HIV reactivation was examined recently in HIV-positive children. The children in whom AIDS progressed rapidly acquired primary HHV-6 infection later than those in whom HIV progressed slowly; however, in the rapid progress to AIDS the HIV viral load did not increase at the outcome of HHV-6 infection. Late in AIDS, HHV-6 detection in PBMCs is reduced, most likely because of T-cell depletion (69). As a rule, the variant A strains are more frequently associated with AIDS patients.

In Vivo Tissue Distribution

In addition to the salivary glands, HHV-6 has been frequently isolated from cultured PBMCs from AIDS patients or children with exanthem subitum or febrile illnesses. This led to the initial definition of HHV-6 as a lymphotropic virus. In lymphocytes, the virus establishes a latent infection, readily monitored by PCR amplification of viral DNA sequences in uncultured PBMCs (47). Furthermore, productive infection has been monitored in some cases by immunohistochemistry (e.g., in CD4+ T lymphocytes) (70). In contrast with the earlier view of HHV-6 as a T-lymphotropic virus, recent investigations detected HHV-6 in many tissues. Despite the wealth of research on the presence of HHV-6 in humans, our knowledge is fragmentary. By immunohistochemical staining, active HHV-6 infection was detected in various cells (e.g., CD68+ cells of the monocyte/macrophage lineage in Kaposi sarcoma [71], epithelial cells and lung macrophages, dendritic cells and macrophages of lymph nodes and infiltrating lymphocytes of organs of unselected patients who died of AIDS, tubular epithelial cells of kidney) and in submandibular glands (67,72). Consistent with this wider host range, HHV-6 DNA sequences were detected in a number of

organs (e.g., skin, spleen, lung, heart, kidney, adrenal gland, esophagus, duodenum, colon, liver, and early bone marrow progenitor cells) from patients who died of heart attack or accidents (47,56,65). Since in numerous studies detection was performed by PCR, latent, persistent, or productive infections were not differentiated, nor was the nature of the infected cells defined. Variant B strains are more frequently found in both PBMCs and solid tissues. Variant A viruses appear predominant in skin and can replicate in primary fibroblast cultures, suggesting a preferential tropism for skin (47). HHV-6 is a brain commensal (see section entitled Neurotropism and Multiple Sclerosis).

In Vitro Tropism

In vitro, HHV-6 infects and replicates at highest titers in PBMCs and CBMCs. In these heterogeneous cultures, susceptible cells are the CD4+ T lymphocytes but also the CD4-CD3+CD8+ and the CD4-CD3- natural killer cells (68). Inasmuch as CD4 expression is not a requirement for susceptibility to HHV-6 infection and soluble forms of CD4 and antibodies to CD4 fail to inhibit virus infectivity (73), CD4 is not a necessary component of the cellular receptor for HHV-6. In addition to primary T lymphocytes, T-lymphocytic lines (e.g., HSB-2, SupT1, Molt3, JJhan, MT-4, ET62) support HHV-6 growth. Viruses of the two variants display different host range, as variant A strains generally do not replicate in Molt3 cells, whereas variant B strains do not replicate in HSB-2 cells. Permissive cells of lineages other than T lymphocytes are the liver cell line HepG2 (74) and a number of human and nonhuman cell lines in which the virus generally grows at very low yields (e.g., cervical cells lines, human primary astrocytes [B variant does not replicate very well] neuroblastoma, human bowel-derived cell monocytes, megakaryocytes endothelial cells, NBL-7 mink lung epithelial cells, and PBMCs of several Macaca species) (75-77). Altogether, in cell cultures as well as in vivo, HHV-6 appears to have a host range wider than initially recognized, extending beyond T lymphocytes. While this is meaningful with respect to studies on the natural history of the infection, the practical use of these cells in the laboratory is hampered by the very low virus yields. Even in the most permissive systems (PBMCs, CBMCs, and T-cell lines), the virus yields are very low. In our experience, CBMC cultures, the most productive cell type, do not yield more that 10^4 infectious units per ml, whereas the titer of a herpes simplex virus type 1 stock is generally as high as 10^9- 10^{10} plaque-forming units per ml.

Neurotropism and Multiple Sclerosis

HHV-6 is probably the most neurotropic virus known. Neuroinvasion has been documented in infants with primary infection, in focal encephalitis, in children and adults with AIDS, in recipients of bone marrow transplants, as well as in immunologically competent children and adults. Challoner et al. (78) reported viral DNA sequences in approximately two thirds of brain specimens and viral antigen expression in a number of cell types (e.g., astrocytes, macrophages, epithelial cells, endothelial cells of blood vessels) at very similar frequencies in specimens from healthy persons and multiple sclerosis patients. Astrocytes were confirmed as a susceptible cell population, although in a subsequent study only samples from AIDS patients were positive (79).

Both variant viruses were detected in the brain of patients who died of causes related or unrelated to HHV-6, which demonstrates that both variant viruses can invade and be harbored in the brain (61,78-82). Although studies on the differential distribution of the two variant groups provided conflicting results (78,83), for HHV-6B, CNS invasion has been documented at primary infection. Instead, for HHV-6A, the time of CNS invasion has not been documented.

A possible correlation between active HHV-6 infection and multiple sclerosis has been the focus of much attention in the past few years. Multiple sclerosis is a severe CNS disease of young adults, characterized by the progressive demyelination of nerves that leads to progressive paralysis and eventually death. The disease appears to be an autoimmune reaction to myelin, the coating of nerve fibers. Viruses have long been suggested as etiologic agents of myelopathies, and DNA sequences from a number of viruses, particularly herpesviruses, have been detected, although not consistently. In addition, since multiple sclerosis is accompanied by a characteristic increase in IgG titer in serum and spinal fluid, antibodies to various viruses (including HHV-6) have been frequently searched for. Even immunologic studies have been

inconclusive, most probably because the increase in antibody response reflects an immune dysfunction or different genetic background together with damage of the blood-brain barrier, rather than an epidemiologic correlation with any given virus.

Studies of HHV-6 infection or reactivation in multiple sclerosis patients have provided controversial results. In initial reports, active infection was suggested by an increase in IgG titer in both serum and spinal fluid but was not confirmed by increase in PCR positivity of PBMCs (84). By representational difference PCR, Challoner et al. (78) found that multiple sclerosis specimens contained HHV-6B DNA sequences. HHV-6B antigen expression was detected at higher frequency in multiple sclerosis plaques than in histologically normal specimens. Nuclei of oligodendrocytes were positive in multiple sclerosis samples (80%) but not in control samples (0%) and were interpreted as a hallmark of the association between active or reactivable HHV-6 infection and the disease. Attempts to reproduce the immunohistochemical results were not successful, and viral expression as documented by reverse transcriptase (RT)-PCR was also negative (85). In favor of a correlation are subsequent findings that the frequency and titer of anti-HHV-6 IgM antibodies are higher in samples from multiple sclerosis patients than from controls (73% vs. 18%) and that the serum DNAemia was specifically positive in multiple sclerosis patients (30% vs. 0%) (86). HHV-6 DNA sequences had been detected in spinal fluid, but not in serum from multiple sclerosis patients (87). Critical interpretation of these data can be summarized as follows. The serologic analyses are difficult to interpret as this disease is characterized by an immunologic dysregulation; therefore, the increase in antibody titer may be a sign of the disease rather than a cause. The PCR data were not confirmed. Thus, no statistical difference was reported in DNA positivity of plaques (32% active vs. 17% inactive plaques) (88), no DNA was detected in serum and cerebrospinal fluid samples (89-91), and no viral RNA was found by RT-PCR in multiple sclerosis brain specimens (85). The differences in PCR results may reflect differences in PCR conditions (e.g., primers, number of cycles, characteristics of the amplified sequences, nature, and conservation of the specimens analyzed) but do not account for the observed discrepancies. Therefore, correlation between active HHV-6 infection and multiple sclerosis is still a controversial issue rather than a firmly established conclusion.

Kaposi Sarcoma

Kaposi sarcoma is a multifocal angioproliferative disease localized predominantly in the skin or mucous membranes and in other visceral organs and lymph nodes. In addition to the classic, iatrogenic, and endemic forms, the disease occurs frequently and aggressively in AIDS patients. Human herpesvirus 8 (HHV-8) sequences were detected for the first time in Kaposi sarcoma specimens (92,93) by representational difference analysis PCR; HHV-8 is being investigated as the possible etiologic agent. Epidemiologic studies had long suggested a viral etiology, and many viruses, including HHV-6 and HHV-7, were detected in Kaposi sarcoma tissues. While neither HHV-6 nor HHV-7 appears to contribute to its etiology, Kaposi sarcoma represents a unique and interesting environment for these viruses, and they may have a role in the progression of the tumor. By immunohistochemistry, HHV-6B has been localized to CD68+ cells of the monocytic macrophage lineage. These cells are either singly infected with HHV-6 or HHV-7 or doubly infected with HHV-6 and HHV-7 (Figure 3) (71). Although some tissues harbor both viruses, albeit in different cells (e.g., lungs and salivary glands), cells doubly infected with HHV-6 and HHV-7 have not been detected in any tissue other than Kaposi sarcoma lesions (94). In addition, in the case of HHV-7, CD68+ cells are a cell type infected, singly or doubly, in no other tissue but in this tumor (71). Data suggest that the particular microenvironment of Kaposi sarcoma lesions, which is rich in chemokines and cytokines, attracts circulating lymphocytes and monocytes that harbor HHV-6 and HHV-7 in a latent or persistent form, induces viral reactivation, and promotes viral growth. In this peculiar environment two unusual situations occur. Viral yields are high for both HHV-6 and HHV-7. This accounts for the likelihood of double HHV-6 and HHV-7 infection, which most likely appears to take place in the tumor itself. HHV-7 tropism is also not restricted to T lymphocytes and extends to CD68+ cells, a lineage not susceptible to HHV-7 infection in other tissues. A predicted chemokine (U83) encoded by HHV-6

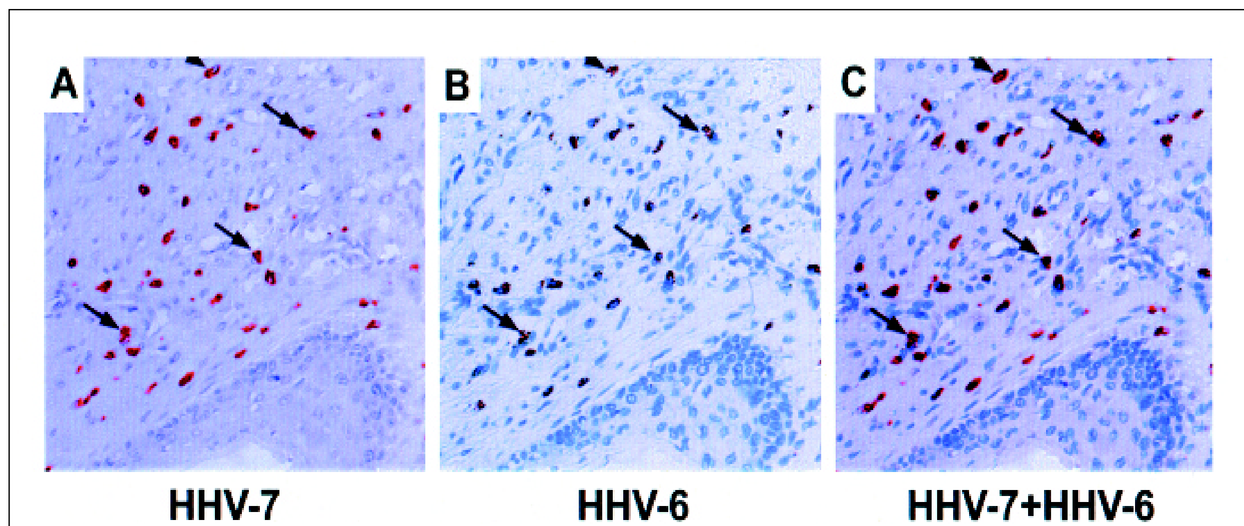

Figure 3. Expression of human herpesvirus 6B (HHV-6B) and HHV-7 antigens in serial sections of Kaposi sarcoma specimens. Panels A-C: In Kaposi sarcoma environment, cells can be doubly infected by HHV-6B and HHV-7: (A) Staining with monoclonal antibody 5E1 to HHV-7-specific antigen pp85; (B) Staining with monoclonal antibody to HHV-6B-specific antigen p101; (C) Overlaid serial sections show colocalization of HHV-6B and HHV-7 (71).

may contribute to dysregulating cellular chemokine expression or signaling. In addition, the virus expresses its own chemokine receptors encoded by the U12, and possibly U51 genes. Once HHV-6 is reactivated and actively replicating, HHV-6 may play a role in tumor progression through these molecules and mechanisms. Different studies detected different variant strains in Kaposi sarcoma tumors (95,96). The reason for this discrepancy is unknown.

Lymphoproliferative and Neoplastic Disorders

Initial isolation of HHV-6 from patients with lymphoproliferative disorders boosted studies on possible association of HHV-6 with proliferative diseases, particularly of lymphoid origin, aimed at showing either a transforming potential of the virus in cell cultures or epidemiologic and molecular relationships between HHV-6 and various types of neoplasia.

In favor of a possible oncogenic potential is the transforming ability of three viral DNA fragments on mouse fibroblast cell lines or human epidermal keratinocytes. One encodes DR7 (97) and the other two encompass the regions spanning U2-U20 and U31-U37. The derived cellular clones were malignant and tumorigenic in athymic mice (98,99). DR7 and the two other loci also contain genes with transactivating activity on the HIV LTR.

Clinical and molecular investigations dealing with HHV-6 and various types of tumors have been reviewed (13). The overall importance of these findings remains controversial, mainly because the criteria for establishing an association between the virus and its oncogenic potential have not been met. Thus, the viral DNA sequences found in a tumor are expected to be the same as those with in vitro transforming potential, and in vitro-transformed cells should be tumorigenic in animals. In a given type of tumor the viral sequences should constantly be the same. In vitro-transformed cells and tumors should express the same viral gene products. The oncogenic potential of the virus should be demonstrated in a suitable animal model (which is lacking for HHV-6). Chromosomal integration of HHV-6 DNA in cells from lymphomas (51,52) may open a new scenario.

CFS

CFS is an illness characterized by memory and attention impairment, muscle and multijoint pain, and unrefreshing sleep and weakness lasting longer than 6 months. The etiology of the disease is unknown, and many viruses have been investigated as possible causing agents. The overall scenario is in a way similar to that of multiple sclerosis. Serologic analysis on the presence of antibody to HHV-6 provided inconclusive data. An increase in IgG and IgM

titer in the sera of a large number of CFS patients (119 of 154) was found relative to that in the control population (77% vs. 12%) (100). However, this was not specific, as an increase in antibodies to other viruses was also detected, reflecting probably an immunologic dysfunction. Molecular analysis showed a higher prevalence of HHV-6A but not HHV-6B or HHV-7 in CFS patients (64,101,102), and HHV-6A could also be isolated from these patients (103). Whether this reflects an association or the consequence of an immune dysregulation remains to be determined.

Conclusions

The epidemiologic and clinical investigations summarized here establish a clear correlation between HHV-6B primary infection and exanthem subitum and between HHV-6 infection/ reactivation and a number of pathologic conditions in immunocompromised patients and transplant recipients. A firm correlation with other diseases remains doubtful. In the case of multiple sclerosis a clearly established correlation may identify patients who might benefit from specific anti-HHV-6 chemotherapy.

Yet another area deserving attention is the state of the virus in healthy people, a key prerequisite to understanding virus behavior in pathologic conditions. We have underlined that, in addition to establishing the true latent infection recognized in earlier studies, HHV-6 persists in the host through a combination of low-level persistent infection of various cells and tissues, a situation similar to that reported recently for HHV-7 (94). Sites of latency may represent a reservoir of the virus, which upon reactivation may feed the sites of persistency.

The pathogenic mechanisms of HHV-6 at the molecular, cellular, and tissue level remain largely obscure. Almost 10 years elapsed between the first isolation of HHV-6 and publication of the sequence of the entire genome. Now, single gene products can be studied in the context of the viral genome and in heterologous expression systems. Although a system for mutagenesis of the viral genome has yet to be developed, the stage is set to ask questions on the molecular mechanisms underlying pathogenicity of the virus. The forthcoming area of research will probably focus on links between the function of single gene products and mechanisms of pathogenesis and virus spread. A key feature of the HHV-6 life-style in the human host is its ability to infect and survive—in a latent or persistent form—in the cells of the immune system, and the pathogenic potential of HHV-6 is linked to its ability to evade immune system control. Analysis of the genomic sequence shows candidates for immune evasion strategies. Yamanishi and colleagues reported that a 7-transmembrane protein encoded by U12 acts as a ß-chemokine receptor (26). As ß-chemokines are key mediators of the immune response, the ß-chemokine receptor may subtract these mediators in a particular microenvironment. The immune evasion strategy must be more complex, as analysis of the viral DNA sequence shows additional candidates, e.g., a predicted chemokine encoded by ORF U83 and a second 7-transmembrane protein—a structural feature typical of chemokine receptors—encoded by ORF U51. This latter protein has a very unusual cell-type-dependent trafficking property (as it is transported to the plasma membrane in infected as well as transfected T lymphocytes) but fails to be transported to the plasma membrane in transfected human monolayer cells (104), raising the possibility that its function is regulated in a cell-dependent fashion through modulation of cell surface expression. Also U51 appears to dysregulate cellular chemokine expression (105). Studies of single gene products will probably lead to the identification of immunodominant proteins and the development of standardized recombinant diagnostic reagents.

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Emergence of a Unique Group of Necrotizing Mycobacterial Diseases

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Although most diseases due to pathogenic mycobacteria are caused by *Mycobacterium tuberculosis,* several other mycobacterial diseases—caused by *M. ulcerans* (Buruli ulcer), *M. marinum,* and *M. haemophilum*—have begun to emerge. We review the emergence of diseases caused by these three pathogens in the United States and around the world in the last decade. We examine the pathophysiologic similarities of the diseases (all three cause necrotizing skin lesions) and common reservoirs of infection (stagnant or slow-flowing water). Examination of the histologic and pathogenic characteristics of these mycobacteria suggests differences in the modes of transmission and pathogenesis, though no singular mechanism for either characteristic has been definitively described for any of these mycobacteria.

Diseases Caused by Emergence of Atypical Mycobacteria

Mycobacterial diseases cause substantial illness and death throughout the world, despite years of public health control efforts. Although most illnesses and deaths are due to tuberculosis (1), particularly in developing countries and in association with the AIDS pandemic (2), diseases caused by nontuberculous mycobacteria (NTM) have had a strong impact on human populations in both developing and industrialized countries (3). Many NTM diseases, such as those caused by *Mycobacterium avium* complex, are considered opportunistic infections in patients with AIDS (4). However, the rates of non-AIDS-associated NTM infections are also increasing (5). Specifically, disease caused by *M. ulcerans*, *M. marinum*, and *M. haemophilum* has increased in both healthy and immunocompromised patients in the last decade. Moreover, these diseases have been reported from previously unaffected geographic areas, which indicates an increase in the geographic distribution of these organisms.

Of these three emerging NTM diseases, Buruli ulcer (BU), caused by *M. ulcerans*, poses the greatest immediate public health threat. Indeed, BU is rapidly becoming the third most prevalent mycobacterial disease, with an impact soon to surpass that of leprosy (6). Although it was first documented in Australia in 1947 (7), the disease was named after the Buruli District of Uganda (8) after an investigation of superficial, ulcerative lesions in Ugandan children. At the time, the disease was sporadically reported throughout Central and West Africa and Australia. In the past decade, incidence of this disease has dramatically increased, with cases now reported in most of sub-Saharan Africa, Mexico, Surinam, Peru, Bolivia, French Guiana, India, sporadically throughout southern Asia, and in Papua New Guinea (6,9). In addition, several cases have been reported in Belgium, Japan, Northern Ireland, and in the United States, resulting from international travel (6,10,11).

A retrospective investigation over a 10-year period in the Daloa region of Côte d'Ivoire was conducted to document increases in the incidence of BU. Cases increased dramatically over a 10-year period, with some villages demonstrating disease rates of 16% of the population at the end of the study (12). Current rates are estimated at more than 22% in some of these villages (6). Increases have also been reported in Australia, with outbreaks of BU during the 1990s in areas where the disease had

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not been previously seen (13). These data most likely underestimate BU occurrence as there are no reliable tools for the surveillance and diagnosis of this disease other than clinical signs and symptoms.

Disease caused by *M. marinum* was observed in clusters of cases between 1930 and 1970, and *M. marinum* was well accepted as a human pathogen before the 1980s. In contrast, *M. haemophilum* was a rarely identified pathogen before 1974. A retrospective study conducted in 1974 reported that, on the basis of epidemiologic evidence, *M. haemophilum* was the causative agent of a syndrome that included mycobacterial adenitis and skin lesions that developed in 29 immunocompetent patients (14). In 1978, the bacterium was identified as the cause of cutaneous ulcerating lesions in a woman with underlying Hodgkin disease (15). Subsequently, *M. haemophilum* has been described as causing cutaneous lesions in persons receiving immunosuppressive therapy after a renal transplant (16).

The occurrence of *M. marinum* and *M. haemophilum* in human disease is likely underreported, as diagnosis of the diseases caused by *M. marinum* and *M. haemophilum* is frequently missed. Nonetheless, confirmed cases of these diseases have been increasing, both internationally (5) and within the United States. A national survey involving 46 state and local laboratory centers, representing 33 states and the District of Columbia, was conducted from 1981 to 1983 to determine the prevalence of NTM diseases. Fifty-three cases of NTM disease caused by *M. marinum* and one case caused by *M. haemophilum* were reported over the 3-year period (17), for a national average number of cases of 40 and 0.76 respectively, per year.

In 1993, a laboratory-based surveillance system (18) began to assess the recent prevalence of NTM disease in the United States. For the *Mycobacterium* module, the population under surveillance included patients in the United States who had a specimen submitted to the state laboratories for evaluation. It is not known what percentage of all mycobacterial isolates this represented. Only one isolate per person was recorded. The geographic distribution and number of cases of *M. marinum* disease (40 states reporting) and *M. haemophilum* disease (9 states reporting), submitted from 1993 to 1996, are presented in Tables 1 and 2, respectively.

Table 1. Laboratory-confirmed cases of *Mycobacterium marinum* in the five regions of the United States (40 states reporting), by year, 1993–1996[a]

	No. cases (%)			
Region	1993	1994	1995	1996
Northeast	21 (14)	40 (22)	34 (23)	28 (18)
Southeast	58 (38)	66 (37)	41 (28)	64 (41)
North central	43 (28)	38 (21)	27 (27)	38 (24)
South central	17 (11)	17 (10)	17 (17)	14 (9)
Mountain	8 (5)	11 (6)	13 (13)	7 (5)
Pacific	5 (3)	7 (4)	15 (15)	6 (4)
Total	152	179	147	157

[a]These data are reported as part of the passive laboratory-based surveillance system using the Public Health Laboratory Information System software developed by the Centers for Disease Control and Prevention and the Association of State and Territorial Public Health Laboratory Directors.

Table 2. Laboratory-confirmed cases of *Mycobacterium haemophilum* in the five regions of the United States (9 states reporting), by year, 1993–1996[a]

	No. cases (%)			
Region	1993	1994	1995	1996
Northeast	0	0	0	0
Southeast	11 (79)	0	0	2 (50)
North central	1 (7)	0	0	0
South central	0	1 (25)	0	0
Mountain	2 (14)	3 (75)	3 (100)	2 (50)
Pacific	0	0	0	0
Total	14	4	3	4

[a]These data are reported as part of the passive laboratory based surveillance system using the Public Health Laboratory Information System software developed by the Centers for Disease Control and Prevention and the Association of State and Territorial Public Health Laboratory Directors.

These data demonstrate that the number of cases of these two diseases in the United States has increased from the past decade, with the estimated national average number of cases of 198 and 35 per year, respectively. In addition, cases of *M. marinum* in several states over the 4-year period of this survey have increased (Table 3) (cases of *M. marinum* had not been reported in Missouri previously [17]). Elsewhere, increases in *M. marinum* disease have been reported throughout the world in temperate climates (5).

In the United States, most cases of *M. haemophilum* disease are still found in the South; however, *M. haemophilum* disease has been described in the New York City metropolitan area (19). In addition, the number

Table 3. Laboratory-confirmed cases of *Mycobacterium marinum* reported by individual states within the United States, by year, 1993–1996[a]

	No. cases (%)[b]			
State	1993	1994	1995	1996
Florida	15 (9.9)	13 (7.3)	13 (8.8)	24 (15.3)
Maryland	15 (9.9)	24 (13.4)	21 (14.3)	22 (14.0)
Minnesota	6 (4.0)	4 (2.2)	6 (4.1)	8 (5.1)
Missouri	2 (1.3)	7 (3.9)	5 (3.4)	9 (5.7)
Utah	3 (1.9)	2 (1.1)	5 (3.4)	4 (2.6)
Virginia	7 (4.6)	13 (7.3)	12 (8.2)	11 (7.0)
Wisconsin	9 (5.9)	8 (4.5)	9 (6.1)	9 (5.7)

[a]These data are reported as part of the passive laboratory-based surveillance system using the Public Health Laboratory Information System software developed by the Centers for Disease Control and Prevention and the Association of State and Territorial Public Health Laboratory Directors.
[b]Percent (%) denotes contribution to cases reported nationally for the year.

of cases of *M. haemophilum* disease in the United States is expanding (Table 2). Thought to occur only in immunocompromised persons (with organ transplant patients and persons with AIDS representing most of the patients) (20), *M. haemophilum* disease was rarely reported even in these populations before 1990. By 1994, 40 cases of *M. haemophilum* disease associated with immunocompromised persons had been reported worldwide (20). However, more recently, a report by Saubolle and colleagues (19) described 10 cases of *M. haemophilum* disease in Arizona (1984 to 1994), which included cases in two otherwise healthy children and three in adults undergoing corticosteroid therapy for rheumatoid arthritis or Crohn disease. Additional cases of *M. haemophilum* disease in otherwise healthy children (21) and adults have recently been observed (M.A. Saubolle, pers. comm.). Elsewhere, *M. haemophilum* disease has been reported in Australia, Canada, France, Israel, and the United Kingdom (19).

Clinical and Histologic Features

Buruli Ulcer

M. ulcerans causes a skin disease commonly known as BU. The incubation period can be highly variable but is generally less than 3 months (22). The ulcers are indolent and necrotizing (9). Systemic signs and symptoms, such as fevers or weight loss, and bacterial superinfection are rare (12,22). Erythema and induration are present at the onset of infection but subside rapidly with the beginning of ulceration. Healing usually takes 4 to 6 months and involves extensive scar formation. This scarring frequently results in deformity, particularly in children, in whom the result can be joint contracture, subluxation, disuse atrophy, or distal lymphedema. Circumferential cicatrization may lead to stunted limb growth. In one series, 26% of patients were left with functional disability of a limb (12). However, death from BU is rare, and no disseminated disease has been reported in either healthy or immunosuppressed persons.

Histologically, *M. ulcerans* produces a circumscribed area of necrosis and (unlike most other mycobacterial pathogens) infected tissues that primarily contain extracellular bacilli, with microcolonies containing large numbers of extracellular acid-fast bacilli (AFB) in the center of the lesions and in association with adipose cells (Figure 1A, B). The effect of AFB at the site of infection can be extensive during the preulcerative phase, with few to no intracellular AFB present (Figure 1B). The lesions are symmetrical with associated coagulation necrosis of the deep dermis and panniculus. The lesions very rarely penetrate beyond the fascia to associate with the underlying muscle. Necrosis occurs extensively beyond the central regions with destruction of capillaries, larger vessels, and adipose cells (Figure 1A) (23). The AFB localize to the adipose tissue (Figure 1B) with necrosis of the adipose tissues occurring at sites distant to the location of the bacilli (Figure 1A) and extensive AFB in all preulcerative nodules and early lesions. The necrosis and damage of the dermis lead to ulceration of the overlying skin. As the ulceration spreads through the panniculus, hypersensitivity granulomas, most likely stimulated by mycobacterial antigens, develop in the dermis and other tissues surrounding the lesions.

M. marinum Disease

M. marinum causes small ulcers or nodules, usually on the extremities (24). The incubation period is approximately 2 weeks to several months (24). These lesions are minimally painful and usually heal in 1 to 2 years without treatment. Main symptoms include slight tenderness and discharge from the necrotic sites. In fewer than 10% of cases, localized lymphangitic

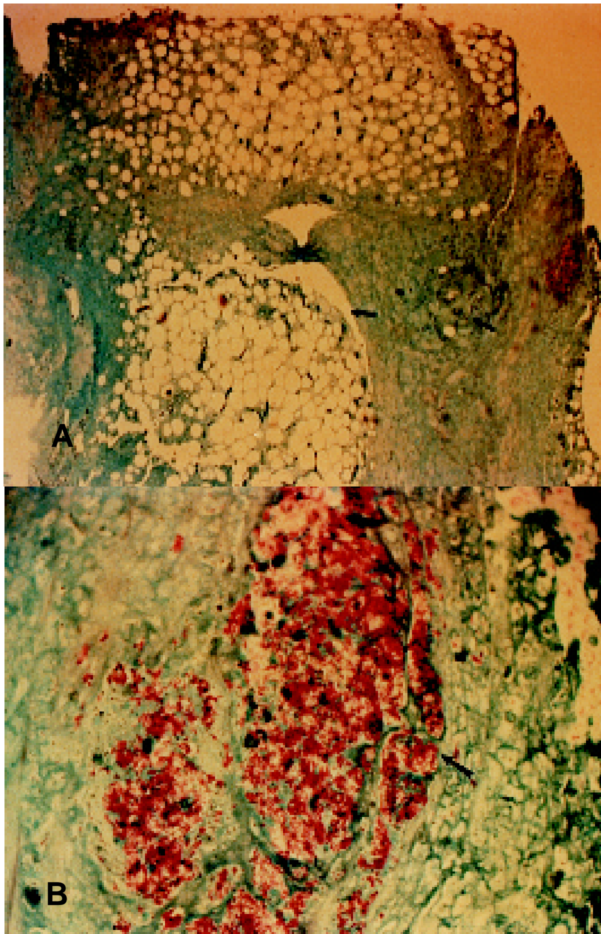

Figure 1. Early- (A) and late-stage (B) disease histopathologic sections of the dermis stained for acid-fast bacilli (AFB) from a patient with a *Mycobacterium ulcerans* infection. In A, arrows indicate necrosis of adipose tissue distant from the location of AFB, and in B, the arrow indicates predominance of extracellular bacilli and microcolonies. Patients' samples were obtained from the study conducted in Côte d'Ivoire (12).
(Photos courtesy of National Center for Infectious Diseases, CDC, Atlanta, Georgia.)

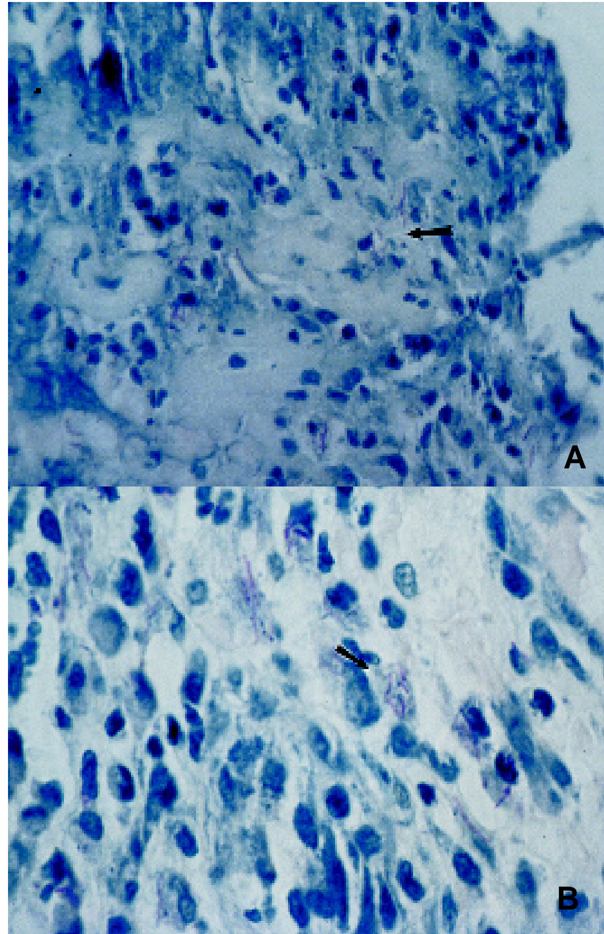

Figure 2 A and B. Active disease histopathologic sections of soft tissue stained for acid-fast bacilli from a patient with a *Mycobacterium marinum* infection. In A, the arrow indicates localized necrosis, and in B, the arrow indicates predominance of intracellular bacilli.
(Slide courtesy of Arthur B. Abt and Leslie Parent, Penn State Geisinger Health System and Pennsylvania State University College of Medicine, Hershey Medical Center, Hershey, Pennsylvania.)

spread is noted, with sporotrichoid lesions and lymphadenitis. These lesions may result in scarring but are less extensive than those caused by *M. ulcerans*; deformity is unusual. Disease in patients with AIDS has been reported (25), and dissemination may occur in the immunosuppressed (26,27).

The bacilli are located throughout the necrotic lesions (Figure 2A), with many bacilli observed as singular rods within cells and vacuoles (Figure 2B). These lesions swell progressively as the infection ensues until nodules are formed. Tissue necrosis usually occurs at small sites within these nodules and is observed only in close proximity to the AFB (Figure 2A). Unlike lesions of *M. ulcerans*, the histopathologic features of early *M. marinum* lesions are similar to those of the lesions observed in pulmonary tuberculosis (24). *M. marinum* lesions generally show nonspecific

inflammation followed by granuloma formation (24). Very few AFB are observed in the lesions themselves; they are present as single or a few bacilli without microcolonies (Figure 2A, B); however, the organism can be cultured from the skin lesion.

M. haemophilum Disease

M. haemophilum generally causes joint and cutaneous infections in immunocompromised patients and lymphadenitis and cutaneous lesions in healthy children (19,28). In addition, a recent report has described cutaneous lesions arising from infection with *M. haemophilum* in two healthy men with no other risk factors for disease (19). Lesions often begin as raised, violaceous nodules, most commonly on the extremities (19,28). In one report, onset of disease occurred approximately 16 months after the onset of AIDS (28). Nodules frequently become erythematous and ulcerated, and recurrence of ulcers may occur in patients in whom complete lesions were not excised (19).

Unlike *M. marinum* lesions, *M. haemophilum* lesions are not sporotrichoid and do not appear to localize to areas above the lymphoid tissues; they are more frequently found above joints, especially appearing around submandibular and cervical joints in infections in children. Mature lesions are often extremely painful, in contrast to mature lesions caused by *M. marinum* and *M. ulcerans*. The organism may also cause septic arthritis or respiratory disease and can be associated with systemic symptoms such as fevers and night sweats. Disseminated disease occurs in severely immunodeficient persons (20). In one report, 9 of 13 immunocompromised patients died, although death may have been due to conditions other than *M. haemophilum* infection (28).

M. haemophilum-infected skin shows minute necrotic foci in the deep dermis surrounded by granulocytes, lymphocytes, monocytes, fusiform cells, and a few giant cells of the Langerhans type (29). Large numbers of bacilli are generally observed both extracellularly and intracellularly as singular cells within these necrotic foci (Figure 3A, B). Histopathologic examinations also reveal poorly formed granulomas within the ulcerated skin lesions (Figure 3B). Similarly, AFB are observed inside and outside the cells and as microcolonies within these granulomas and in the surrounding tissue (Figure 3A, B).

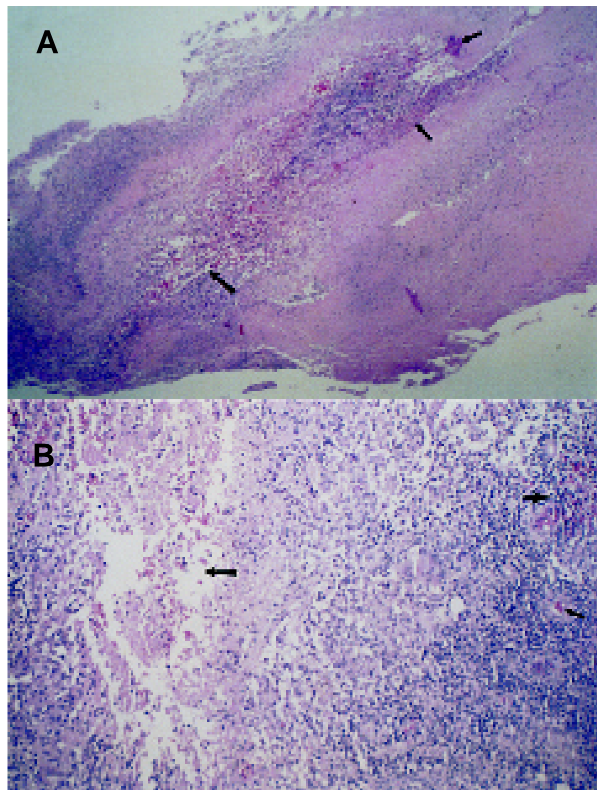

Figure 3 A and B. Active disease histopathologic sections of the epidermis stained for acid-fast bacilli from a patient infected with *Mycobacterium haemophilum*. In A and B, the arrows indicate localized necrosis and presence of intracellular and extracellular bacilli and microcolonies and the presence of loose granulomas.

(Slide courtesy of Michael A. Saubolle, Good Samaritan Regional Medical Center, Phoenix, Arizona, and Department of Medicine, University of Arizona School of Medicine, Tucson, Arizona.)

Pathogenesis

One hallmark of most diseases caused by mycobacteria is the ability of the bacilli to grow within host cells. *M. haemophilum* and *M. marinum* grow prolifically within fibroblast, epithelial cells (Figures 2, 3) (30,31) and macrophages (29,32). In contrast, *M. ulcerans* primarily forms extracellular microcolonies within necrotic tissues, is rarely found within host cells, and disrupts macrophages and adipose cell monolayers in vitro in lieu of growing within these cells (33; our unpub. obs.). Rastogi et al. (34) showed that although *M. ulcerans* would infect and persist in murine macrophages after 4 days, no intracellular growth occurred. Others have demonstrated that culture filtrates from *M. ulcerans* sup-

pressed phagocytosis of the bacilli and speculated that this in vitro suppression of phagocytosis is the reason that the bacilli are only rarely observed within host cells in human disease (33).

The necrosis in the skin lesions of all three mycobacterial infections suggests a secreted or somatic cytotoxin or other necrotic bacterial component.

Buruli Ulcer

The pre-ulcerative and early ulcer stages of BU are characterized by a central zone of microcolonies of AFB surrounded by a larger zone of necrotic tissue with no evidence of host-derived inflammatory exudates that might contribute to cytotoxicity (35). Further, culture filtrates from *M. ulcerans* produce a cytotoxic effect on cultured fibroblasts (35). This material simulated clinical and histopathologic changes similar to those in BU when injected into guinea pigs (36). An initial analysis of the culture filtrates identified a high molecular weight phospholipoprotein-polysaccharide complex that retained the ability to produce a cytotoxic effect on cell monolayers (37). Others have ascribed the cytotoxic effect of *M. ulcerans* to a low molecular weight lipid (742 daltons) in the filtrates (38). Fractionation of the culture filtrates and observation of the effects of each fraction on cultured L929 fibroblast cells initially identified a lipid as the cytotoxic component. Further purification and analysis of this lipid did not induce cell death, but rather arrested the cellular growth (38).

Some suggest that the factors secreted by *M. ulcerans* may also possess immunosuppressive properties indirectly contributing to the destruction of human tissue (33). Others believe that the necrosis of tissue is due primarily to infarction, with no contribution from cytotoxic bacterial factors (23). Thus, the overall cytotoxic effects demonstrated by *M. ulcerans* in human disease may result from multiple factors. No study has addressed whether cytotoxic or immunosuppressive factors are released from *M. ulcerans* during the early, active, or late stages of infection; the mechanisms by which such factors might act on host tissues are also not known.

M. marinum Disease

In *M. marinum* infection, bacilli are capable of invading and replicating within cultured macrophages and epithelial cells (31). Intracellular growth of *M. marinum* is limited at temperatures above 33ºC, as the bacilli do not grow intracellularly at 37ºC. In one study, temperature-dependent growth also correlated with cytotoxicity of macrophage monolayers, but no evidence of secreted toxins was noted from supernatants of these infected tissue cultures. The investigators suggested that intracellular growth and a faster growth rate at 33ºC probably caused cytotoxicity (31).

M. marinum produces skin lesions in animal models without prior induced immunosuppression. Intravenous injection of *M. marinum* into normal mice caused skin lesions similar to those observed in humans, but no dissemination of the organism occurred (40). Dissemination of *M. marinum* was induced only when mice or leopard frogs (*Rana pipiens*) were subjected to conditions that lower the immune response, such as lower body temperatures or treatment with hydrocortisone (40,41). A strain adapted to an optimal growth temperature of 37ºC in broth culture produced immediate disseminated disease when injected into the foot pads or tail veins of normal mice (40), demonstrating that once temperature restriction was removed as a barrier to growth, the bacilli quickly disseminated regardless of immune status of the mice.

M. haemophilum Disease

A putative cytotoxin from *M. haemophilum* has not been reported, even though the presence of such a toxin is suggested by histopathologic examinations of infected tissues and in vitro tissue culture studies (30). In particular, an epithelial cell tissue culture model demonstrated that *M. haemophilum* induced substantial cytotoxicity in epithelial cells at 33ºC but not at 37ºC, even though the bacilli grew extracellularly in coculture with epithelial cells at 37ºC (30). Filtered supernatant from 33ºC infected tissue cultures produced identical cytotoxic reaction when layered onto fresh monolayers. However, unlike from *M. ulcerans*, broth medium from bacterial culture was not cytotoxic. These studies suggest that a cytotoxin may be produced only upon infection of epithelial cells growing at 33ºC. Intracellular growth occurred only during infections at 33ºC, even though electron microscopy showed that the bacilli were capable of invading these cells at 37ºC (30). This temperature-specific cytotoxicity and intracellu-

lar growth mimic the clinical signs of infection in humans. The bacilli grow in cooler, superficial regions of the body where primary tissue destruction occurs; subsequently, the bacilli may spread to deeper, warmer tissues of the host, where little tissue disruption is observed and granulomas form around the infected areas (19).

Systemic symptoms have been reported but were not likely caused by a cytotoxin released by the bacteria (19). Animal studies suggest that immunosuppression leads to disseminated *M. haemophilum* disease. The development of skin lesions and dissemination by *M. haemophilum* do not occur when the bacilli are injected intravenously into healthy mice. However, upon inoculation with *M. haemophilum*, mice treated with steroids to induce immunosuppression develop skin lesions and disseminated disease similar to human disease (39).

Epidemiology

Buruli Ulcer

The reservoir of *M. ulcerans* is unknown. The organism has only been recovered from lesions of humans or, in one case, a koala (42), and there has been only one report of person-to-person spread (9). Thus, environmental exposure, either by direct inoculation or an insect vector, is the likely route of infection. Epidemiologic studies suggest that proximity to water sources, such as freshwater lakes or rivers, predisposes to disease, but specific contact with water that might lead to transmission of the bacteria has not been identified. In fact, one study showed that BU was more common during the dry season (43). Cultures of water near BU-endemic areas have not yielded *M. ulcerans* (9,12,43,44), though testing of water samples by polymerase chain reaction found *M. ulcerans* DNA (44,45). Soil also has been considered a possible reservoir, but the organism has not been isolated from soil samples.

BU is primarily a disease of children, with the highest rates found in children ages 2 to 14 years (12). Boys and girls are equally affected. In some areas, women also have an elevated risk of BU (12). No data are available on disease rates by race, but the disease has been reported in all racial groups. Although reported in persons with HIV (46,47), no predilection for BU has been noted in HIV-infected persons or other immunodeficient patients, despite the substantial rates of HIV in many BU-endemic areas (9,48).

M. marinum Disease

Water is the source of infection by *M. marinum* in humans (24). Recovered from unchlorinated swimming pools and salt and fresh water aquariums associated with cases of disease, the organism is also a common pathogen of fish; however, water is likely the reservoir, and fish are a susceptible host. The organism may be transmitted through minimal trauma or abrasions to the skin. Person-to-person spread has not been well documented, and no geographic localization of the disease has been noted (41).

The mean age of persons with *M. marinum* disease is 35 to 42 years (17,24), although cases have been reported in all age groups. Caucasians, men, and urban residents are at highest risk. Like BU, the disease is not associated with immunosuppression and is rare in AIDS patients, even though cases are common in industrialized countries where AIDS is prevalent (25-27).

M. haemophilum Disease

Unlike *M. ulcerans* and *M. marinum*, most *M. haemophilum* infections occur in immunocompromised patients (20). There is scant evidence of person-to-person spread of *M. haemophilum*, similar to that observed for *M. ulcerans* and *M. marinum* infections. *M. haemophilum* disease also appears to be acquired from the environment, but the reservoir is unknown; the organism has been isolated only from humans with disease (20,49). A case-control study that included cases apparently acquired through nosocomial transmission did not find common epidemiologic elements (28). Another study considered the role of aerosolized pentamidine in the spread of *M. haemophilum*. In this study, six patients at a cancer center contracted *M. haemophilum* disease after prophylactic therapy with aerosolized pentamidine, with two of these patients having *M. haemophilum* pneumonia. The role of this therapeutic treatment in exposure could not be clarified either, as numerous patients receiving this therapy did not contract *M. haemophilum,* nor was the organism recovered from the reconstituted pentamidine or

water used before and after nebulization (19). Because cases have occurred in persons residing near the ocean or large lakes, water has been suggested as a possible reservoir (20,50). This conjecture, however, does not explain the increase in the number of cases observed in the southwestern United States, particularly Arizona (19).

The median age of persons with *M. haemophilum* disease is 31 years, and 80% were immunosuppressed (20). Two thirds of cases were in men, and most were in Caucasians.

Factors Relevant to the Mode of Transmission

Mycobacteria are widespread in the environment, particularly in aquatic reservoirs. In one survey, more than 67% of water specimens collected from natural, treated, and animal-contact sources contained mycobacteria, including *M. marinum* (50). Mycobacteria also are commonly found in soil. Wolinsky and Rynearson (51) identified at least one *Mycobacterium* species from 86% of the samples they collected from several locations. *M. haemophilum* was not recovered from any samples in this survey, probably because the culture methods used were not suitable for the growth of this *Mycobacterium*.

M. ulcerans

M. ulcerans grows slowly at all temperatures between 25ºC and 37ºC, although greater proliferation is observed during growth at temperatures between 30ºC and 33ºC. No colony variants have been reported, and the bacterium apparently has a shorter doubling time in a medium enriched with fatty acids, a phenomenon consistent with bacilli in lipid-rich areas surrounding sites of infection (6,9).

Singular lesions involving areas of the body most susceptible to trauma (i.e., upper and lower extremities) are frequently observed, and direct inoculation is the most plausible mode of transmission for *M. ulcerans* infection. Numerous case histories document skin trauma and abrasions preceding the onset of BU (9,12). Others propose that an insect vector may transmit *M. ulcerans* (54), although supporting evidence for this hypothesis has not been reported.

M. ulcerans infections may be linked to environmental disturbances (9,12,23,43). The first reported cases of *M. ulcerans* infection occurred 2 or 3 years after severe regional flooding and continued intermittently until 1950 (7). In 1978, there again was severe flooding in this area, and again approximately 2 years later, infections occurred, first in koalas and later in humans. Cases of BU were also observed on the east side of the Victoria Nile in Uganda between 1962 and 64 (43); this outbreak was also likely associated with severe flooding in the region caused by heavy rains. As in the outbreak observed in Australia, 2 or 3 years elapsed between the flooding in Uganda and the first cases in the region. In Togo, infection in children was related to seasonal flooding of rivers in proximity to the local village (55). Cases were reported in one area in Liberia after swamp rice was introduced to replace upland rice. This introduction was associated with the construction of dams on a major river and the artificial extension of wetlands (56). In addition, recent cases described in Côte d'Ivoire occurred primarily in association with farming activities near the main river (12). Therefore, a common feature of *M. ulcerans* disease is that infected persons often reside near swampy areas, river valleys, or lakes and coastal areas.

After a flood or some other environmental disturbance, mycobacteria may be washed from their normal habitat into draining rivers or lakes. Given favorable circumstances, such as relative stream stagnation, temperatures of 27ºC to 33ºC, low salinity, low pH, and the presence of adequate nutrients, survival and growth of the organism may be enhanced. With the temperatures that are reached in the tropics, moderate temperatures in the surface water and in moist silt beside lakes may be sufficient to sustain the growth of *M. ulcerans* during the daytime for most of the year (57). Moreover, *M. ulcerans* could be dispersed from a water environment in a fashion analogous to that documented for aquatic *M. avium*, where droplet aerosolization of the organism may result in infection (50,58), also suspected in recent BU cases in Bairnsdale, Australia. Most of the patients infected with *M. ulcerans* resided in a small region near one of the lakes but showed no history of direct contact with the water (57). This type of airborne dispersal would also explain the acquisition of BU in tree-living koalas that may be exposed to contaminated aerosols generated in the adjacent lake system (59). Additionally, airborne dis-

persal might explain the prevalence of disease on a geographic continuum in all countries bordering the Gulf of Guinea in West Africa (6). Finally, spray aerosolization of *M. ulcerans* in recycled sewage water used to irrigate a golf course has been proposed as the route of infection for another series of cases in Australia (13).

M. marinum

Unlike that of *M. ulcerans* and *M. haemophilum*, the etiology of *M. marinum* disease is well known. Of the three, *M. marinum* has the clearest association with water as the source of the infection (60). In 1926, *M. marinum* was first isolated and identified as the cause of saltwater aquarium fish deaths (61). Tuberculoid skin lesions in users of a swimming pool in 1939 and 1954 and granulomatous mycobacterial disease in freshwater fish in 1942 also were ultimately attributed to *M. marinum* (62). Since then, a variety of skin infections due to this organism have been observed around the world, and names such as "mariner's TB," "aquarium granuloma," and "swimming pool granuloma" have been coined to describe the disease as well as the source of the infection. However, when swimming pools are properly chlorinated, this association has all but disappeared (60). Nevertheless, virtually any water source and water-related activity is a potential risk, including tending aquariums (62), fishing (63), skin diving (62), and a number of other water-related activities (64).

M. haemophilum

M. haemophilum is fastidious, grows slowly, requires supplemental iron, and has a lower incubation temperature for growth than most other mycobacteria. This organism is usually grown on chocolate agar, on egg-based media, or on Middlebrook media containing 15 µg/mL to 25 µg/mL ferric ammonium citrate, 0.4% hemoglobin, 60 µmol/L hemin, or on X-factor (52). The temperature growth range is 25°C to 35°C, but the optimal incubation temperature is 32°C (52). Because of these requirements, *M. haemophilum* cannot be isolated by the standard techniques used in clinical laboratories, and its fastidiousness may account for the lack of isolation from environmental sources and patient specimens.

The ecology of *M. haemophilum* is poorly understood, and the reservoir and modes of transmission still need to be elucidated. However, one study describing *M. haemophilum* isolates from different patients in the same hospital that had identical fingerprint patterns (53) supports the possibility that the patients were exposed to a common source, such as water. Additional evidence that *M. haemophilum* grows over a wide pH range (49) and can use chelated iron (52), characteristics common to other aquatic bacteria, suggests an environmental niche. This organism survives in cold water (our unpub. obs.) and is resistant to chlorine (50). Several researchers have suggested using frogs as models for the study of *M. haemophilum* systemic disease, as they (or other amphibians) have cooler body temperatures and thus could be environmental sources for this organism (15).

Possible modes of transmission for infection with *M. haemophilum* include inhalation, ingestion, and skin inoculation. Since most infections occur on the skin, direct inoculation may be most likely. However, patients were no more likely than healthy persons from the area to recall injuries or skin conditions before the onset of symptoms (19), and patients generally did not report cutaneous injuries before illness (19). The isolation of *M. haemophilum* from sputum and lung tissue suggests the possibility of respiratory transmission; however, respiratory therapies, exposure to irritants, or previous respiratory infections have rarely been associated with infection (19). Interestingly, an early animal model demonstrated that *M. haemophilum* can spread to the skin through dissemination in the blood after intravenous inoculation, suggesting bloodborne transmission (39). This study was performed on prednisolone-treated mice challenged intravenously with *M. haemophilum;* skin lesions (predominantly on the cooler regions of the body) developed in 12 of 30 mice.

This collective evidence illustrates a defined reservoir and mode of transmission for *M. marinum* infections, and, although similar evidence is described for *M. ulcerans* and *M. haemophilum*, the precise mode of transmission of these infections remains undefined.

Conclusions

We have discussed the emergence of three skin diseases and presented the pathophysiologic, epidemiologic, and environmental characteristics that may contribute to their emergence.

Disease caused by *M. ulcerans* and *M. marinum* is primarily in immunocompetent persons, while the emergence of *M. haemophilum* disease is primarily in immunosuppressed persons. All three mycobacteria are thought to be acquired by inoculation during contact with contaminated water, suggesting that changes in the environment may be contributing to their emergence.

Histologic studies demonstrate that *M. ulcerans* has an apparent association with adipose tissue, is not observed within host cells in vivo, and does not grow intracellularly within cultured macrophages. In contrast, *M. marinum* and *M. haemophilum* grow intracellularly within cultured macrophages and epithelial cells and have apparent associations with epithelial tissues in vivo, suggesting that different cellular tropisms occur between these species after the dermis is initially infected. None of these organisms readily cause disseminated disease in immunocompetent humans, and this restriction may be related to the optimal growth temperature of these mycobacteria (temperatures similar to that of the human dermis), although all three can grow at warmer temperatures, albeit to a lesser extent in vitro. *M. marinum* colony variants that grow best at 37°C in vitro overcome this apparent temperature restriction in vivo and disseminate in animals, further demonstrating that while temperature-restricted growth likely limits the spread of disease, this restriction can be surmounted by acquired factors. *M. haemophilum* can become a disseminated disease in immunosuppressed persons, suggesting that the organism can also overcome temperature restrictions in a favorable environment.

The common source of infection for these three mycobacteria is likely water (it is the definitive source for *M. marinum).* As with the source of infection, the mode of transmission has been clearly defined as inoculation after skin abrasion for *M. marinum*, while no clear association with skin abrasion has been demonstrated for infection with *M. ulcerans* or *M. haemophilum* in humans. Experimental inoculation of all three organisms into the skin of animals produces pathologic effects similar to those in humans. However, epidemiologic studies on *M. ulcerans* and *M. haemophilum* and disease and laboratory experiments with these two organisms in animals suggest different modes of transmission than *M. marinum*, including possible aerosol transmission with subsequent hematogenous spread to the dermis.

Finally, comparisons of the pathogenic and histopathologic characteristics of these three diseases suggest differences in the levels of virulence, mechanisms of pathogenesis, and modes of transmission. As we learn more about the ecology, epidemiology, and pathogenesis of these unique mycobacteria through improvements in culture techniques, diagnostic tests, and continued laboratory research, we may be able to identify contaminated environments contributing to the source of these infections and elucidate the mode of transmission. Such information is essential if we are to develop strategies to prevent the further emergence of these diseases.

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Respiratory Diseases among U.S. Military Personnel: Countering Emerging Threats

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Emerging respiratory disease agents, increased antibiotic resistance, and the loss of effective vaccines threaten to increase the incidence of respiratory disease in military personnel. We examine six respiratory pathogens (adenoviruses, influenza viruses, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae,* and *Bordetella pertussis*) and review the impact of the diseases they cause, past efforts to control these diseases in U.S. military personnel, as well as current treatment and surveillance strategies, limitations in diagnostic testing, and vaccine needs.

Respiratory infections, the most common cause of acute infectious disease in U.S. adults (1), are also the leading cause of outpatient illness and a major cause (25% to 30%) of infectious disease hospitalization in U.S. military personnel (2,3). Because of crowded living conditions, stressful working environment, and exposure to respiratory pathogens in disease-endemic areas, military trainees and newly mobilized troops are at particularly high risk for respiratory disease epidemics (2, 4-6). For example, before vaccines were used, more than 80% of military trainees had respiratory infections, and as many as 20% were hospitalized during the 2 months of recruit training (7). Although respiratory disease control is improved, epidemics continue to occur, and respiratory disease in military trainees continues to exceed that in U.S. civilian adults (Figure 1). The recent loss of adenovirus vaccine (types 4 and 7) production, changes in the susceptibility of pathogens to antimicrobial drugs, and emerging respiratory pathogens threaten to increase the military population's vulnerability to respiratory diseases.

We review the changing epidemiology and control of six major respiratory disease pathogens of special concern to the military.

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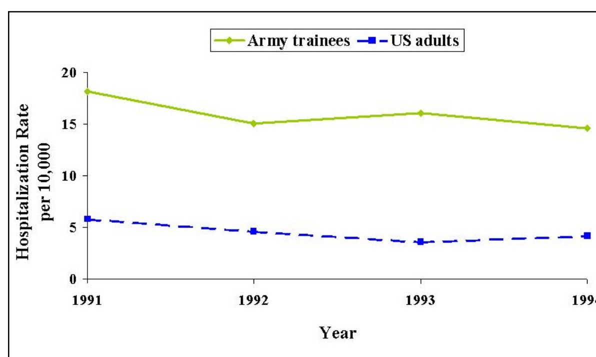

Figure 1. Hospitalization rates for acute respiratory disease per 10,000 persons, 1991 to 1994: U.S. army recruits vs. young adults in U.S. nonfederal hospitals. U.S. army recruit estimates are converted from percentage febrile acute respiratory disease rates per 100 trainee-week figures (8). On average, recruits were 19 years old. U.S. national nonfederal estimates were taken from first-listed diagnoses with the International Classification of Diseases codes 460 to 466 (9) among persons of ages 15 to 44 years (10-13).

Adenoviruses

Respiratory disease agents discovered in adenoidal tissue in U.S. soldiers in the 1950s were associated with rhinitis, pharyngitis, conjunctivitis, pneumonitis, and atypical pneumonia and were subsequently designated as adenoviruses (14). In 1958, adenoviruses caused hospitalization of an estimated 10% of military recruits (15). Adenoviral disease was highest during winter, accounting for 90% of all recruits

hospitalized with pneumonia (16,17) and 72% of all respiratory disease (17). Military recruits had a greater chance of acquiring adenoviral infections than similar civilian populations, with most infections occurring during the first 3 weeks of military training (16,18,19). Of the 47 adenoviral serotypes, types 4 and 7 accounted for most military respiratory disease epidemics. A 1965 study of a typical epidemic at Fort Dix, New Jersey, established the need for vaccines (20).

In 1971, the Department of Defense (DoD) began routine use of live, enteric-coated types 4 and 7 vaccines, which have remained very effective (6). Vaccine development for other serotypes that cause only infrequent epidemics was begun, but no vaccine became licensed. Recently, the sole manufacturer of the adenovirus type 4 and type 7 vaccines ceased production, so neither vaccine is available. The unavailability of adenovirus vaccines threatens a sharp increase in numbers of acute respiratory disease epidemics in the military, especially among recruits (6). Recently, two recruit centers where the vaccines were not available had large acute respiratory disease epidemics (21,22).

The ecologic and pathologic features of adenoviruses in military populations are poorly understood (23,24). Most available surveillance data are more than 20 years old (7,20). To better understand the distribution of adenovirus serotypes, risk for infection, and agent dynamics following vaccine loss, triservice adenovirus surveillance has been established at five military training centers (Figure 2) (25). Early data indicate that types 4 and 7 vaccines remain effective, but nonvaccine serotypes are prevalent and should be considered in new vaccine development strategies. More than 55% of 3,212 throat cultures from symptomatic trainees from October 1996 to May 1998 yielded adenoviruses. Most prevalent were types 4 (46%), 7 (32%), 3 (13%), and 21 (5%). Among trainees with acute respiratory infection symptoms, nonvaccinated personnel were at greater risk of having a culture positive for adenovirus types 4 and 7 (odds ratio = 41.2; 95% confidence interval = 18.7 to 113.2) than vaccinated personnel. Capability to isolate and identify adenoviruses has improved, but simple rapid molecular diagnostic techniques have not yet been developed.

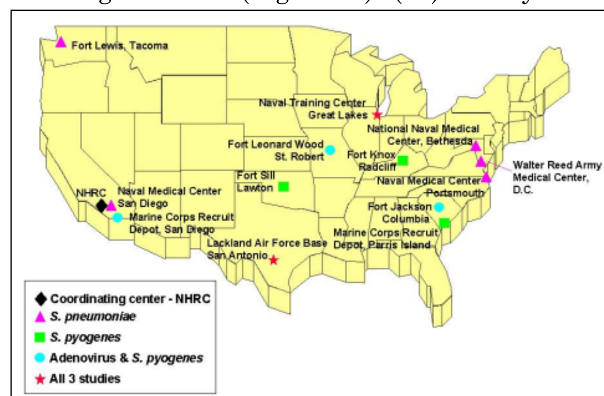

Figure 2. Department of Defense medical treatment facilities and recruit training camps participating in surveillance for emerging respiratory disease pathogens: invasive *Streptococcus pneumoniae* (typing and antibiotic sensitivity studies); *Streptococcus pyogenes* (typing and antibiotic sensitivity studies); and adenovirus (typing studies).

Influenza

Since an annual influenza vaccine policy was adopted for active-duty personnel in the 1950s, massive influenza epidemics have largely ended. However, the potential for illness and death due to new viral strains remains. During the last 3 months of 1918, an influenza A pandemic affected 106,897 (18.8%) of 569,470 navy personnel, with an estimated case-fatality rate of 4.5%. The case-fatality rate was particularly high among military trainees, especially those who had pneumonia. For example, during a 30-day period beginning in September 1918, 9,623 (21.5%) of 44,605 navy trainees (Illinois) had influenza, and 924 died; the case-fatality rate was highest (48%) among those with pneumonia (26). At autopsy, streptococcal organisms were often associated with pneumonia, which suggests that pathogens in the training camps may have exacerbated the influenza illnesses and deaths during this pandemic.

Even with annual use of influenza vaccine, laboratory-based surveillance is critical. During February 1996, a U.S. navy ship with a 600-person crew had an estimated 42% influenza A attack rate, although more than 95% of the crew had received the annual influenza vaccine (K. Earhart, pers. comm.). The annual vaccine for that winter (A/Johannesburg/33/94-like [H3N2] and A/Texas/36/91-like [H1N1]) did not protect against the A/Wuhan/359/95 [H3N2] strain that infected the crew.

The recent outbreak of H5N1 influenza A in Hong Kong prompted a review of capability to detect new influenza strains (27); only the air force was conducting a laboratory-based surveil-

lance program (28). Since the Hong Kong outbreak, a cooperative global influenza surveillance network has been formed. Worldwide, more than 20 medical treatment facilities and laboratories from all services are collecting influenza isolates for typing (Figure 3) (29). Additionally, in the United States, military training sites at high risk for influenza are monitored so that epidemics might be quickly detected. This early warning system allows public health officials to modify vaccine antigens, use antiviral drugs, and take other measures to reduce illness.

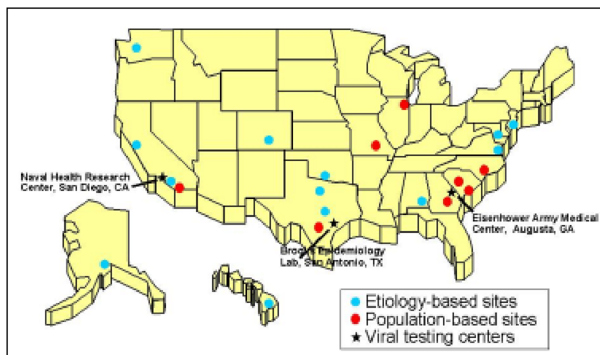

Figure 3. Military sites in the United States participating in Department of Defense influenza surveillance. The focus of surveillance at etiology-based sites is to determine the viral causes of influenzalike illnesses; the focus of population-based sites is to closely monitor for influenzalike illness epidemics.

Streptococcus pneumoniae

Before penicillin was introduced, complications of *S. pneumoniae* infections were frequent and often fatal. Large epidemics of pneumonia occurred in crowded military populations, particularly after influenza outbreaks, especially during winter. In 1918, a 1-month epidemic of *S. pneumoniae* in a military camp in Illinois resulted in 2,349 hospital admissions with a 50% death rate (30). From the 1960s to the 1980s, military epidemics of pneumococcal disease were very rare. However, in recent years, military pneumococcal epidemics have occurred in southern California (31), in North Carolina (32), and among a ship's crew in the Mediterranean Sea (4).

S. pneumoniae infections have various clinical features, including pneumonia, meningitis, empyema, bacteremia, conjunctivitis, sinusitis, arthritis, and otitis media. Since the introduction of penicillin, epidemics of respiratory disease caused by *S. pneumoniae* are much less frequent but remain a threat. To counter outbreaks, the military has used mass prophylaxis with benzathine penicillin G (1.2 million units) intramuscularly (31). However, the efficacy of this intervention and its impact on antibiotic resistance have not been fully evaluated (33). In 1991, the Armed Forces Epidemiological Board recommended pneumococcal vaccine (23-valent polysaccharide) for populations at high risk for *S. pneumoniae* infection. However, because of the cost and uncertainty about efficacy in healthy young adults, the vaccine is given only to trainees at one marine corps installation.

During the last 20 years, penicillin-resistant *S. pneumoniae* (intermediate and highly resistant strains), as well as multidrug-resistant strains, have been reported with increasing frequency throughout the world. Recently, investigators from Korea reported that 70% of 131 clinical civilian pneumococcal isolates were penicillin-resistant (34). On the basis of limited surveillance data, the public health threat of penicillin-resistant *S. pneumoniae* to U.S. military personnel and their dependents is increasing (35). Walter Reed Army Medical Center, Washington, D.C., reported an increase in the percentage of penicillin-resistant *S. pneumoniae* isolates from 0% in 1990 to 36.2% in 1994 (36).

In winter 1989-90, an outbreak among marine trainees at Camp Pendleton, California, resulted in 128 reported cases of pneumonia, one of the largest epidemics since the development of antibiotics. The epidemic triggered mass prophylaxis with benzathine penicillin G and administration of pneumococcal vaccine (31). Soon after, other smaller epidemics of pneumococcal pneumonia occurred among U.S. army rangers (32) and among the crews of two navy ships in Italian waters (4). As we write (March 1999), another pneumococcal pneumonia outbreak among army trainees is under epidemiologic investigation. These recent pneumococcal epidemics may be evidence of a changing epidemiologic threat. Pneumococcal pneumonia, which was checked when antibiotics became available in the 1950s, seems to have reemerged.

Increasing antibiotic resistance and epidemics prompted surveillance for invasive

S. pneumoniae disease (Figure 2). Early data from patients hospitalized in military hospitals in the United States are consistent with data from civilian U.S. populations. On average, 35% of isolates have full or partial resistance to penicillin (35,37). Thus far, nearly all invasive isolates are of types included in the 23-valent vaccine. A cost-effectiveness analysis projected that using this vaccine among new navy and marine corps personnel would result in a lifetime savings of approximately $9 million (38).

Streptococcus pyogenes

U.S. military populations have frequently had large *S. pyogenes*–caused epidemics of pharyngitis and acute rheumatic fever, accompanied by other concomitant diseases, such as pneumonia, sepsis, polymyositis, necrotizing fasciitis, scarlet fever, and glomerulonephritis (5,39). Historically, because of cramped living conditions, military recruits have been at high risk for streptococcal disease (2,5,39,40). Illness was especially high in World War II, with the navy reporting approximately one million streptococcal infections and more than 21,000 cases of acute rheumatic fever (5,41).

In 1948, Massell et al. (42) reported that the treatment of acute pharyngitis infection with oral penicillin prevented acute rheumatic fever. Further studies confirmed the effectiveness of a single intramuscular injection of benzathine penicillin G in preventing a broad range of acute and chronic sequelae of streptococcal infections (5,40,43). These early successes led to mass antimicrobial prophylaxis with benzathine penicillin G in training populations at high risk to interrupt and prevent outbreaks of acute disease and their sequelae (44). This control strategy was generally very effective. However, a 1989 epidemic of *S. pyogenes* pharyngitis among marine corps trainees demonstrated that benzathine penicillin G prophylaxis for nonpenicillin-allergic trainees alone might not protect against epidemics in closely contained populations, especially those with longer training periods, as unprotected penicillin-allergic recruits may serve as *S. pyogenes* reservoirs. This finding led to the navy's adoption of oral erythromycin as prophylactic therapy for penicillin-allergic recruits (39,45). Another study has shown that 500 mg of azithromycin taken orally each week is, by serologic evidence, an effective prophylactic intervention against *S. pyogenes* (33).

Since the development of antibiotic prophylaxis, civilian and military epidemics of *S. pyogenes* disease have declined and then reemerged (39,46,47). Epidemics of acute rheumatic fever have occurred throughout the United States (46-48). In addition, an estimated 10,000 cases of severe *S. pyogenes* disease, such as necrotizing fasciitis and streptococcal toxic shock, occur nationwide each year (49-52). Increases in invasive streptococcal disease among some U.S. populations have been attributed to changes in the prevalence of virulent strains of *S. pyogenes*(53).

Although antibiotic prophylaxis remains effective, *S. pyogenes* persists as a leading cause of bacterial respiratory illness among military personnel (5,39,47,48,54). Risk factors associated with *S. pyogenes* infection include recent entry to the military, crowding, lack of prophylaxis, close contact with an *S. pyogenes* carrier, and close contact with a trainee who has not received antibiotic prophylaxis (39).

Prophylactic use of oral erythromycin or azithromycin may promote macrolide resistance among endemic streptococci. The Naval Medical Center, San Diego, California, found 5(10%) of 50 consecutive clinical isolates collected during March and April 1997 resistant to erythromycin. While frequently reported in Europe and Japan, macrolide resistance has been uncommon in U.S. military populations (T. Ferguson, R. Haberberger, pers. comm.) and infrequent in isolates from civilians in the United States (55).

Triservice surveillance has been established to define antibiotic resistance patterns and determine which serotypes of *S. pyogenes* are causing clinical disease (Figure 2). Data from eight sentinel military medical treatment facilities will be used to monitor resistance and develop alternate prophylactic strategies, rapid diagnostic tests, and vaccines.

Mycoplasma pneumoniae

During World War II, acute pneumonia in military personnel was frequently milder than lobar pneumonia. Chest radiographs showed substantial pulmonary involvement, yet patients did not have high fever, pleuritic chest pain, or rigors characteristic of pneumonia caused by *S. pneumoniae*. In 1943, these infections were

recognized as primary atypical pneumonia, which accounted for an estimated 68% of atypical pneumonias among marine trainees (56) and infected as many as 44% of recruits over a 3-month training period (57). In 1944, samples from a patient with atypical pneumonia showed *M. pneumoniae* (57,58), and soon thereafter, *M. pneumoniae* was identified as an important cause of acute respiratory disease in U.S. military personnel (59).

A common cause of pharyngitis and bronchopneumonia, *M. pneumoniae* may also cause fulminant pneumonia, cardiac disease, arthritis, dermatologic conditions, and central nervous system disease (60). Crowded military populations are at particularly high risk for infection. In the 1970s, up to 57% of U.S. recruits had evidence of acute infection (61), and from the 1960s through the 1990s, as many as 56% of pneumonia cases among recruits were due to *M. pneumoniae* (62-64). Because culture and diagnostic tests for *M. pneumoniae* are not commonly available at military facilities, *M. pneumoniae* is often not recognized, and ineffective antibiotics are prescribed (62).

Few options are available for combating *M. pneumoniae* epidemics. More than 25 years ago, several studies suggested that preexisting antibody titers might prevent infection (65,66), and vaccine candidates were tested with mixed success (64,67,68). In 1965, preventing disease with a 10-day course of oxytetracycline (69) (4 times a day) among close contacts was successful but impractical. More recently, weekly oral azithromycin (500 mg) had a 64% protective efficacy (by serologic tests) against *M. pneumoniae* in U.S. marines (33).

Reliable diagnostic tests and enhanced surveillance efforts are needed to assess the epidemiology and impact of *M. pneumoniae* on military populations. With the exception of serologic tests, few rapid diagnostic tests are commercially available.

Bordetella pertussis

Before vaccines were available, *B. pertussis* caused considerable illness in children. With the effectiveness of whole-cell childhood vaccines, disease incidence increased among older children and adults, whose childhood vaccine immunity had waned (70-74). *B. pertussis* infection in adults, while generally mild (75), can be incapacitating. No pertussis vaccines are available for adults.

B. pertussis also affects military populations; a 1989 study of marine trainees who reported 7 or more days of cough showed that 18% had acute *B. pertussis* infection (73). The potential for military epidemics of *B. pertussis* is demonstrated by outbreaks among other confined populations, such as those receiving general or institutionalized medical care, which have attack rates as high as 91% (76,77). Infection in adults is often difficult to verify since culture and polymerase chain reaction diagnostic tests may be negative (73). While often used epidemiologically, serologic methods are not standardized, nor are they routinely performed by clinical laboratories (78). Hence, many epidemics are monitored by clinical case definitions.

Some clinicians have observed a prophylactic benefit in administering oral erythromycin to close contacts of patients (78). However, erythromycin prophylaxis is not without side effects, and its value has been questioned (76,79). New acellular pertussis vaccines, now approved only for use among infants and children, are being studied for use in adults (80).

Research and Disease Control

Trainees entering military service receive influenza vaccine and adenovirus types 4 and 7 vaccines when available. Mass antibiotic chemoprophylaxis is also often used to prevent acute respiratory disease and control epidemics, particularly those caused by *S. pyogenes* infections. After initial training, military personnel receive annual influenza vaccine and periodic tuberculosis screening (Table).

Almost all respiratory illnesses, including pneumonia, are treated empirically (4,62), often with penicillin or a macrolide (62). Without accurate laboratory diagnoses and an early warning system to detect changes in acute respiratory disease rates and antibiotic resistance, more respiratory disease epidemics are likely to occur in military populations. A Global Emerging Infections Surveillance and Response System has been established to address this problem. Surveillance data will be used to direct acute respiratory disease research, training, and education. Under the system, DoD has recently established modest surveillance programs for influenza, adenovirus, *S. pyogenes*, and

Table. Current capacity to control respiratory pathogens at most military medical treatment facilities, United States

Pathogen	Culture	Rapid diagnostic tests	Prophylaxis	Vaccine[a]
Streptococcus pyogenes	Available	Antigen detection available and used	Benzathine penicillin G (5), erythromycin (45), or azithromycin (33)	Needed
Streptococcus pneumoniae	Available but not sensitive	Needed	Azithromycin (33)	Available but seldom used among military personnel
Mycoplasma pneumoniae	Not available	Serologic tests are available	Azithromycin (33)	Needed
Influenza	Not available[b]	Needed	Amantadine	Vaccine available and routinely used
Adenovirus	Not available[b]	Needed	Not available	Types 4 and 7 vaccines effective but not available
Bordetella pertussis	Available but not sensitive	Needed	Erythromycin prophylaxis is of questionable value (79)	Needed

[a]While a number of civilian populations, such as the institutionalized, may have similar needs these vaccine needs are particularly urgent for crowded military trainees.
[b]Some medical treatment facilities have access to culture support.

S. pneumoniae at a number of U.S. military recruit training camps and special facilities, in collaboration with other federal, state, and civilian organizations. Recruit sites were chosen for their long history of respiratory disease epidemics and the possibility of monitoring the impact of mass antibiotic prophylaxis. Tertiary referral medical centers were chosen to participate because they were more likely to detect unusual and antibiotic-resistant strains of respiratory pathogens. Limited samples of clinical influenza A, adenovirus, *S. pyogenes,* and *S. pneumoniae* isolates are being studied. However, new diagnostic tools and vaccines are still needed (Table).

Conclusions

Military personnel, because of crowding and unique stressors, are subject to respiratory disease epidemics. Their risk often exceeds that of their civilian peers. Adenovirus, influenza virus, *S. pyogenes*, *S. pneumoniae*, and *B. pertussis* are particularly problematic. Pathogen control measures, many of which were developed more than 20 years ago, are threatened by loss of vaccine production, changes in pathogen virulence, changes in pathogen antibiotic sensitivity, changes in population immunity, and lack of laboratory infrastructure to identify respiratory disease pathogens and evaluate new diagnostic and control measures.

Strong, laboratory-based surveillance programs are needed to quickly identify new problems. The surveillance programs must be supported by fast, accurate diagnostic laboratory tests. Surveillance data must then be used to direct the development and evaluation of new interventions, particularly vaccines.

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Q Fever in Bulgaria and Slovakia

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As a result of dramatic political and economic changes in the beginning of the 1990s, Q-fever epidemiology in Bulgaria has changed. The number of goats almost tripled; contact between goat owners (and their families) and goats, as well as goats and other animals, increased; consumption of raw goat milk and its products increased; and goats replaced cattle and sheep as the main source of human *Coxiella burnetii* infections. Hundreds of overt, serologically confirmed human cases of acute Q fever have occurred. Chronic forms of Q fever manifesting as endocarditis were also observed. In contrast, in Slovakia, Q fever does not pose a serious public health problem, and the chronic form of infection has not been found either in follow-ups of a Q-fever epidemic connected with goats imported from Bulgaria and other previous Q-fever outbreaks or in a serologic survey. Serologic diagnosis as well as control and prevention of Q fever are discussed.

Q fever, a widespread zoonosis recognized as a clinical entity in 1937 (1), is caused by the obligate intracellular parasite, *Coxiella burnetii*. The disease is endemic worldwide, occurring in different geographic regions and climatic zones (2). New Zealand is probably the only large country without Q fever (3). The principal vectors of *C. burnetii* are ticks, which transmit the agent to wild animals (causing wildlife coxiellosis) or to domestic animals (creating the livestock reservoir of *C. burnetii)* (4). The most important reservoirs in nature are small wild rodents, but infection was also demonstrated in insectivores, lagomorphs, carnivores, ungulates, ruminants, marsupials, monkeys, bats, birds, and even reptiles and fish (5,6). Infected domestic animals (cattle, sheep, and goats but also pet animals, especially cats), frequently with persistent and subclinical coxiellosis, represent the main source of *C. burnetii* infection for humans, who become infected by direct contact with these animals, by environmental contamination (from animal excrements), and (indirectly) through processing or consuming animal products. Human infection is acquired most often by the inhalation of contaminated aerosols but may also occur through the digestive tract, through skin trauma, or by sexual contact. Mother-to-fetus transmission may also occur (7).

C. burnetii may cause acute and chronic forms of Q fever in humans, though in many cases infection is asymptomatic and confirmed by serologic diagnosis only (8). The acute form of Q fever manifests usually as a flulike illness or atypical pneumonia, but often it has a protean character with a clinical picture resembling that of nearly any infectious disease (9). Whereas acute Q fever is usually self-limited, chronic Q fever is a serious and often fatal illness with death rates exceeding 65% (10). Illness occurs months to years after the acute infection in 1% to 11% of patients and usually manifests as endocarditis. Infections of arterial aneurysm or prosthesis; bone infection; pseudotumor of the lung; hepatitis; cutaneous, musculoskeletal, or renal involvement; and placentitis in pregnancy with miscarriage are also possible (11).

The first human cases of Q fever in Europe appeared in the Balkans during World War II when strange, febrile, influenzalike infections, named Balkangrippe, were observed among German troops (12,13). Similar infections occurred among allied troops during operations

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in the Mediterranean area (14,15). In the 1940s, Q fever was recognized in Romania (16), Greece (17), and Bulgaria (18). The Balkans thus became the territory in which *C. burnetii* could circulate in nature, be transmitted to humans, and be spread to other parts of Europe; for example, Q fever was probably introduced to Slovakia through infected sheep from Romania (6).

Q Fever in Bulgaria

Though the first human Q-fever cases in Bulgaria were described as early as 1949 (18), thorough epidemiologic and epizootologic studies started later in connection with the unification of land and livestock farms into state premises and agricultural cooperative units. Concentration of domestic animals (especially cows and sheep) and conditions favoring circulation and maintenance of *C. burnetii* in nature (ticks, reservoir animals) turned the country into a huge natural focus of Q fever. The occurrence of *C. burnetii* infection in different parts of the country was 6% to 100% in sheep, 5% to 31% in cattle, and 7% to 34% in goats (19), as confirmed serologically by complement fixation (CF). Infestation of ticks with *C. burnetii* reached 26% in southwest and 22% in northeast Bulgaria (20).

The situation changed dramatically in the 1990s as the large state premises and cooperative farms collapsed and the number of cows and sheep decreased (e.g., sheep from 8 million in 1990 to 3 million in 1997). As individual farmers started to raise goats for easily accessible food, the number of goats increased from 430,000 in 1990 to more than 1 million in 1997. Moreover, the proportion of sera containing antibodies to *C. burnetii* from domestic animals from five regions of Bulgaria in 1996 to 1997 also changed; more (90% of 140) samples from goats than from sheep (73% of 118) tested positive. At the same time, the rate of infestation of *Dermacentor marginatus*, *Ixodes ricinus*, and *Rhipicephalus sanguineus* with *C. burnetii* as demonstrated by the hemocyte test (21) was also very high (25 of 29 ticks tested were infected).

These changes also influenced the occurrence and seasonality of human Q-fever cases in Bulgaria. Until 1990, more than 20 Q-fever outbreaks occurred in several regions of the country, some with hundreds of patients (e.g., 725 cases near Knyezha from 1982 to 1985 [22] and as many as 630 cases in Pavlikeni in 1985 only). These Q-fever epidemics among farmers explain the sharp peak of Q fever incidence in 1985 (Figure 1). A sudden drop and continual decrease thereafter could reflect a return to a nonepidemic situation; the gradual increase in the 1990s was probably associated with an increase in the number of goats (Figure 1). Depicting the seasonality of the disease, the number of goats shifted from winter and spring months (December to May) with a peak in January in the 1980s to spring and summer months (March to August) with a peak in May and June in the 1990s (Figure 2).

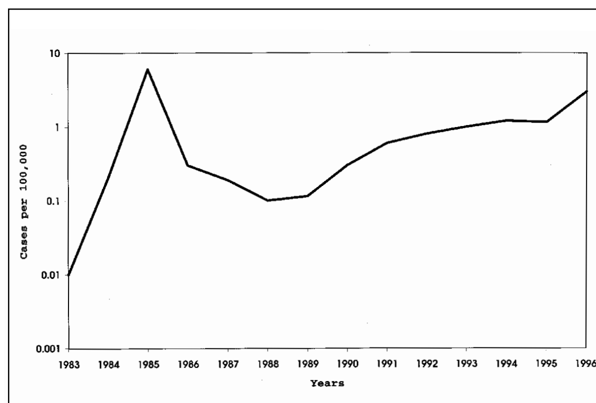

Figure 1. Incidence of human Q fever in Bulgaria, 1983–1996.

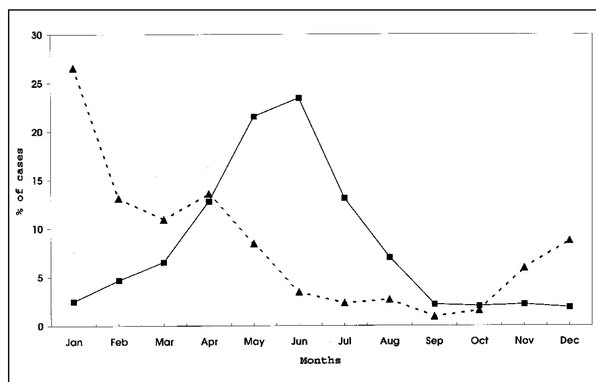

Figure 2. Seasonal distribution of acute human Q-fever cases in Bulgaria in 1983 to 1989 (dashed line) and 1990 to 1996 (full line).

Several factors probably contributed to this shift. 1) A change in insemination practices. Until 1990, artificial insemination of ewes was carried out in early autumn, but in the 1990s, this practice was abandoned. 2) Sheep deliver in January and February, whereas goats deliver in March and April. 3) Herds of cattle and flocks of

sheep, though concentrated in larger numbers, were separated from most of the population and served as the source of *C. burnetii* infection usually among farmers and others exposed to animals and their products. 4) An increased number of goats have been kept in very close contact with goat owners and their family members. 5) Goats have been pastured daily (from March until October), so they cross the streets of villages and small towns twice a day. Thus, at present in Bulgaria, goats (rather than sheep or cattle) seem to be an important source of *C. burnetii* infections for humans. Not only have great numbers of goat owners with their families been exposed to *C. burnetii* (when tending the goats or consuming their unpasteurized milk products), but also occasional bystanders could contract *C. burnetii* infection from exposure to the contaminated aerosols created from excrements of the goats, as they repeatedly passed through villages and small towns to their pastures or from exposure to infectious particles generated during parturition or abortion. For example a Q-fever outbreak in Val de Bagnes, Switzerland, which affected 415 persons, occurred 3 weeks after 12 flocks of sheep descended from the Alpine pastures to the valley (8).

The largest Q-fever outbreak was registered in Panagyurische (central part of southern Bulgaria) in the 1990s, after an influenza epidemic (end of 1992, beginning of 1993). From January to June 1993, a second epidemic wave with more than 2,000 cases of an acute flulike respiratory illness and bronchopneumonia occurred. Atypical pneumonia was diagnosed in 589 cases by X-ray examination, and 254 patients were admitted to the regional hospital. Q fever was confirmed serologically only at the end of the epidemic. Of more than 500 persons who recovered from Q-feverlike disease, 60% were seropositive; several laboratories confirmed significant titers (from 80 to 640 in CF and microimmunoflourescence [MIF] tests) of antibodies to phase II *C. burnetii*. Though most of the patients and those with positive serologic tests were adults 20 to 59 years of age, high positivity was also noticed in children <6 years of age (19%) and in persons 7 to 19 years of age (23%). Most of the patients were not employed in agriculture or the processing of animal products. Serologic examination of domestic animals by CF test gave similar results for goats (26% of 969) and sheep (28% of 421). However, sheep did not seem to serve as a source of *C. burnetii* infection—they went to pastures far away from the town from February to March. The highest number of human Q-fever cases was observed from April to June when goats delivered their kids and sporadic goat abortions occurred.

A Q-fever outbreak occurred in Panagyurische again in April to June 1995 as evidenced serologically in 78% of 89 patients admitted to the local hospital with bronchopneumonia. Acute Q fever was diagnosed in 28 (31%) on the basis of seroconversion or fourfold rise of antibody titers. The results indicate that Q fever appears to be endemic in the Panagyurische area (with seasonal spring occurrence) and goats are probably the main source of human infection. Nevertheless, such a seasonal Q-fever occurrence has not been restricted to this area of Bulgaria (Table) but occurred in all the regions (Ichtiman and Elin Pelin in the West, Stara Zagora in the South, Blagoyevgrad in the Southwest, Vratza in the Northwest, and Varna in the Northeast) of the country in which it had been followed. Of 252 patients with bronchopneumonia and acute flulike symptoms, 66% (46%-100%) had antibodies to phase II *C. burnetii*

Table. Phase II-*Coxiella burnetii* antibodies in patients with bronchopneumonia and acute respiratory symptoms, Bulgaria

Town (region)	Testing period	No. positive/ No. tested[a]	% positive (% acute)
Ichtiman (West)	April-June 1995	19/21	90.5 (38)
Elin-Pelin (West)	April 1995	9/9	100.0 (44)
Blagoyevgrad (Southwest)	March-June 1996	26/44	59.1 (34)
Blagoyevgrad (Southwest)	March-June 1997	24/30	80.0 (60)
Stara Zagora (South)	March-June 1997	24/52	46.2 (28)
Vraca (Northwest)	March-June 1997	37/61	60.7 (13)
Varna (Northeast)	Feb-March 1997	28/35	80.0 (14)
Total		167/252	66.3 (29)

[a]Sera from Ichtiman and Elin-Pelin were examined by complement fixation test, remaining sera by microagglutination. All sera were from patients with bronchopneumonia, except those collected in Varna, which were from patients with acute respiratory flulike symptoms. Sera with titers ≥ 10 in either test were considered serologically positive. Acute Q-fever diagnosis was based on seroconversion or fourfold rise of antibody titers.

detected by CF or microagglutination (MA) tests. Acute Q fever was confirmed in 73 (29%) patients tested (13% to 60% in different regions). In all these regions, sheep, cattle, and goats have been raised not only in villages surrounding the above-mentioned towns, but also in their suburbs.

Similar results were observed when testing human sera from the serum bank. Of 224 randomly chosen sera collected in nine localities (Gabrovo from the North, Razgrad and Dobrich from the Northeast, Sofia from the West, Blagoyevgrad from the Southwest, and Stara Zagora, Pazardzhik, Haskovo, and Sliven from the South of Bulgaria), 87 (38%) reacted positively with phase II *C. burnetii* in MA or MIF tests. Serologic positivity varied from 6% in Sliven to 60% in Blagoyevgrad, except for Razgrad, where none were positive (however, only four sera were tested).

Chronic Q-fever cases manifesting as endocarditis were confirmed serologically by high titers from 640 to 1 ml of phase I- and phase II- *C. burnetii* immunoglobulin (Ig)G antibodies in MIF, by demonstration of specific immunofluorescence in the cuts of aortal valves, and by *C. burnetii* isolation from the replaced prosthesis in three patients before 1990 (23). Two additional cases of Q-fever endocarditis were diagnosed serologically by MA and MIF tests from 1996 to 1997.

Antibodies to phase II *C. burnetii* by MA were found in 16 of 18 aborting women with titers of 10 to 320, which indicates the possibility of acute Q-fever infection during pregnancy. In two cases, in paired sera collected in 23-day intervals, a shift from the titer of 160 to seronegativity (titer <10) was observed. Even though abortion tissues were not cultured or tested for *C. burnetii*, these findings deserve further study, since the possible adverse effects of *C. burnetii* infection during pregnancy has also been suggested by other authors (24).

Q Fever in Slovakia

In Slovakia, Q fever has been known since 1954 when outbreaks occurred among agricultural workers who contracted the infection from sheep imported from Romania and among workers of a textile plant who were exposed to contaminated imported cotton (6). From that time until the 1980s, the waves of epizootics and small epidemics appeared in factories processing cotton, wool, and hides from Mongolia and China, in a sheep farm with imported breeding rams from England, and in various agricultural premises often connected with excursions of workers to cattle or sheep farms in which *C. burnetii* infections could have occurred. Veterinary and serologically uncontrolled movement of cattle within the country also contributed to the establishment of domestic coxiellosis (25). Some areas of the southern part of central Slovakia became a natural focus of Q fever, with the *D. marginatus* tick as the main vector of *C. burnetii* (6).

Since the 1980s, only sporadic cases of Q fever have been reported from different parts of the country, though almost 3% of approximately 7,000 ticks collected in all districts of Slovakia were found (by the hemocyte test) to harbor *C. burnetii*, and attempts to recover *C. burnetii* from pooled positive ticks resulted in the isolation of 10 virulent *C. burnetii* strains from five ticks, mostly *I. ricinus* species (26). On the other hand, *C. burnetii* strains isolated from cow milk were of lower virulence for guinea pigs and mice (Kováčová et al., submitted for publication). Circulation of such low virulent strains among livestock and large-scale vaccination of cattle by inactivated phase I-*C. burnetii* corpuscular vaccine (one subcutaneous dose consisting of 500 µg of highly purified *C. burnetii* cells) carried out in the 1970s and 1980s, together with improved veterinary control of domestic animal transport within the country, could explain a decrease in the occurrence of human Q fever in Slovakia. This explanation is supported by results of a serologic survey (carried out from 1989 to 1996) for Q-fever antibodies in groups of farmers or in patients with suspected *C. burnetii* infection. Of 21,197 human sera tested, 655 (3%) reacted with phase II- *C. burnetii* antigen in the CF test (until 1992) or (later on) in enzyme-linked immunosorbent assay (ELISA). Acute Q fever (as individual or clustered cases) was diagnosed in 23 sera, not including 113 from the Q-fever epidemic discussed below, on the basis of seroconversion or IgM antibody detection. During the same period, phase-II *C. burnetii* antibodies were detected in 11% of cattle and in 3% each of sheep and goats.

Improved veterinary control of domestic animal transport within the country, however, cannot exclude the possibility of introducing *C. burnetii* infection through imported domestic

animals or raw materials not tested properly. The use of CF, which is much less sensitive to Q-fever antibodies than other serologic tests (e.g., ELISA [27]), to screen the goats imported to Slovakia from Bulgaria is unsatisfactory, as confirmed in 1993 by the largest reported Q-fever epidemic in Slovakia (28). The epidemic started suddenly during the spring as an outbreak of respiratory infection in inhabitants of a village in West Slovakia. A total of 113 persons were affected from the beginning of March until May 18, as confirmed serologically (seroconversion, detection of IgM antibodies, and high phase-II antibody titers, respectively) by CF, MA, MIF, and ELISA. Of 42 patients admitted to the hospital, 33 had atypical pneumonia (diagnosed by X-ray examination), and 27 had hepatic involvement (diagnosed on the basis of the increased values of liver transaminases). As many as 103 were male patients who used to visit the local pub, in which they contracted infection by the aerosol created from the heavily contaminated garments of boys tending aborting goats. *C. burnetii* infection in incriminated goats was confirmed serologically (46 of 216 goat sera tested were positive by ELISA) and by seroconversion in mice inoculated with spleen, lung, and liver suspension from an aborted kid.

In contrast to the situation in Bulgaria, a 4-year follow-up of patients from this Q-fever epidemic did not result in clinical or serologic confirmation of any chronic form of the disease (Kováčová et al., submitted for publication). In addition, evidence of chronic Q fever was obtained neither in the serologic survey carried out in Slovakia from 1989 to 1996, nor in testing of more than 200 patients with chronic cardiovascular disease (some of them exposed to *C. burnetii* infection through their work). Similarly, observation of patients from other Q-fever epidemics (including those with 98 cases in a cotton-processing plant in nearby Southern Moravia in 1980 [29]) was also negative. Whether this can be explained by *C. burnetii* strains of different virulence circulating in Bulgaria and Slovakia, respectively, remains to be seen, though in the latest Q-fever epidemic in West Slovakia, Bulgarian *C. burnetii* strains were presumably involved. However, whereas *C. burnetii* strains of tick and domestic animals origin isolated in Slovakia may differ, no data are known on the virulence of Bulgarian strains. A total number of human *C. burnetii* cases can also be important. Whereas in Bulgaria more than 1,000 patients were affected, in Slovakia tens of human cases occurred, so the probability of developing the chronic form of Q fever in Slovakia was lower. The small number of patients in Slovakia could also be explained by earlier diagnosis and proper antibiotic treatment at the early stage of infection. The patients' history, e.g., previous rheumatic disease, should be also taken into consideration.

Lessons Learned from Q-Fever Outbreaks in Bulgaria and Slovakia

Epidemiologic and serologic investigations in Bulgaria and Slovakia indicate that an increase in human Q fever in Bulgaria in the 1990s and Slovakia in 1993 was associated with goats. The data on the propensity of goats to transmit *C. burnetii* to humans from Greece, Cyprus, France, the United States, and even a trans-Pacific cargo ship transporting dairy goats, were summarized by Lang (4). More recently, a cluster of human *C. burnetii* infections associated with exposure to vaccinated goats and their unpasteurized products was reported from France (30). Goats may pose a threat to human health as a source of *C. burnetii* infection in every country in which they are raised extensively and are in close contact with humans. However, Q fever can also be contracted from other sources of infection and has been, even in Bulgaria and Slovakia.

Reporting of Q fever in a given territory depends on the attention of public health authorities and the availability of diagnostic methods. Apart from *C. burnetii* isolation (mainly in cell cultures by a shell-vial method [31] or direct detection, preferably by polymerase chain reaction [32]), these diagnostic methods are based mostly on serologic tests. Sensitivity of serologic tests for screening Q-fever antibodies increased from CF to MA and from MIF to ELISA (27). The cut-off values for individual tests may differ between laboratories and antigens used; for CF and MA tests, 1:8 and 1:16 serum dilutions were acceptable (in Slovakia) and for either test 1:10 serum dilution was acceptable (in Bulgaria). For a more sensitive MIF and ELISA allowing also detection of immunoglobulin classes, diagnostic titers were set at the phase-II IgG $\geq$ 200 and phase-II IgM $\geq$ 50 in MIF (33) and at $\geq$ 128 for the IgM and IgG phase-I responses, but $\geq$ 512 for the IgM and $\geq$ 1,024 for the IgG

response to phase II *C. burnetii* in ELISA (34), respectively. Serologic diagnosis of acute Q fever relies on seroconversion from negativity to positivity or at least fourfold rise of phase-II antibodies in paired (acute- and convalescent-phase) serum samples and demonstration of IgM antibody response or high titers (e.g., ≥ 128 in CF and 200 in MIF) of phase-II antibodies in a single serum sample. For diagnosis of chronic Q fever, high titers (i.e., ≥ 200 in CF and ≥ 800 of in MIF) of phase I antibodies, occurring rarely and in low titers in acute Q-fever cases, are required (9).

Q-fever control and prevention measures have been reviewed (35). Apart from the thorough control of imported domestic animals, raw materials, and movement of domestic animals within a country, prevention measures should include adequate disinfection and disposal of animal products of conception and strict hygienic measures in cattle, sheep, and goat farms; plants processing products of these animals; boiling or pasteurization of milk at 62.8°C for 30 minutes or at 71.7°C for 15 seconds; and vaccination. At present, three types of Q-fever vaccine are available for human use: a Formalin-inactivated whole-cell phase-I *C. burnetii* vaccine used in Australia (36), a chloroform-methanol residue subunit of phase-I *C. burnetii* recommended by American authors (37), and Q-fever chemovaccine (a soluble subunit vaccine obtained by treatment with trichloroacetic acid of phase-I cells) developed and used in Romania (38) and the former Czechoslovakia (39). For vaccination of domestic animals, corpuscular phase I (in Slovakia) or phase II (e.g., in France) were used. The fact that phase-II vaccine did not protect goats from shedding *C. burnetii* in milk (30) confirmed that an effective Q-fever vaccine should consist of or be prepared from phase-I *C. burnetii* (40). Efficient recombinant vaccines, however, should also be pursued.

Mass vaccination of cattle in Slovakia in the 1970s, followed by selective vaccination of cattle in serologically positive herds and elimination of positive reactors in the 1980s could lessen not only distribution of *C. burnetii* among domestic animals, but also its transmission to humans. However, absence of vaccination of domestic animals in Bulgaria could contribute to the maintenance of *C. burnetii* and therefore to increased possibility of human infection, though basic natural conditions for circulation of this agent in either country have been similar. Moreover, gradual changes in agriculture in Slovakia during the 1990s resulted in reduced numbers of cattle and sheep but not in the dramatic increase in goat numbers seen in Bulgaria after the collapse of state farms and cooperative units. One can conclude that in Bulgaria there is a permanent threat of more Q-fever outbreaks unless preventive measures, including improvement of veterinary services and vaccination of domestic animals, particularly goats, are established. In Slovakia, because of surveillance, veterinary control, and vaccination of domestic animals, the situation is much better; however, attention should still be paid to avoid introduction of *C. burnetii* by imported animals and raw materials and the possibility of coxiellosis outbreaks among domestic animals and consequently Q fever in humans.

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Adhesins as Targets for Vaccine Development

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Blocking the primary stages of infection, namely bacterial attachment to host cell receptors and colonization of the mucosal surface, may be the most effective strategy to prevent bacterial infections. Bacterial attachment usually involves an interaction between a bacterial surface protein called an adhesin and the host cell receptor. Recent preclinical vaccine studies with the FimH adhesin (derived from uropathogenic *Escherichia coli*) have confirmed that antibodies elicited against an adhesin can impede colonization, block infection, and prevent disease. The studies indicate that prophylactic vaccination with adhesins can block bacterial infections. With recent advances in the identification, characterization, and isolation of other adhesins, similar approaches are being explored to prevent infections, from otitis media and dental caries to pneumonia and sepsis.

The ultimate aim of any vaccine is to produce long-term protective immune responses against a pathogen. These responses include systemic humoral antibodies that neutralize invasive organisms and cytotoxic T cells, which are required to clear certain infections, particularly chronic viral infections such as HIV (1). Helper T-cell and cytokine responses also influence humoral and cellular immune responses (2). For most bacteria and viruses, the first encounter with their host involves attachment to a eukaryotic cell surface, which results in colonization of the host prior to disease. In such cases, induced antibody responses at the mucosal surface could prevent attachment and abrogate colonization. The ideal target for such antibodies—surface proteins known as adhesins—mediate microbial attachment to host tissue (3). We review recent advances in the identification, isolation, and purification of adhesins (and putative adhesins) that could serve as vaccines to elicit such responses. Studies of at least one adhesin, the FimH protein from uropathogenic *Escherichia coli*, show that anti–adhesin antibodies can block microbial attachment and subsequent disease. Furthermore, while specific induction of immune responses along the mucosal surface by mucosal immunization may have its advantages (4-7), the FimH studies demonstrate that immunoglobulin (Ig) G antibodies alone, which transudate into secretions after parenteral vaccination with the FimH adhesin, are sufficient to block colonization and infection.

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The Role of Adhesins in Microbial Pathogenesis

To initiate infection, bacterial pathogens must first be able to colonize an appropriate target tissue of the host (8,9). This tropism (ability to gain access to a niche within the body), in association with the ability of the bacterium to breach mucosal barriers and invade the host, distinguishes pathogenic from commensal organisms. Colonization begins with the attachment of the bacterium to receptors expressed by cells forming the lining of the mucosa (Figure 1A, 1B). Certain species of bacteria are restricted in terms of the hosts and tissues they infect and the diseases they cause. In many cases, tropism for specific tissues has been corroborated in the laboratory by in vitro binding assays with isolated epithelial cells collected from sites of infection or from infection-prone hosts.

Attachment is mediated by adhesin proteins; bacterial lectins are the most common type of adhesin among both gram-negative and gram-positive bacteria (3,10-12). Adhesins, such as the

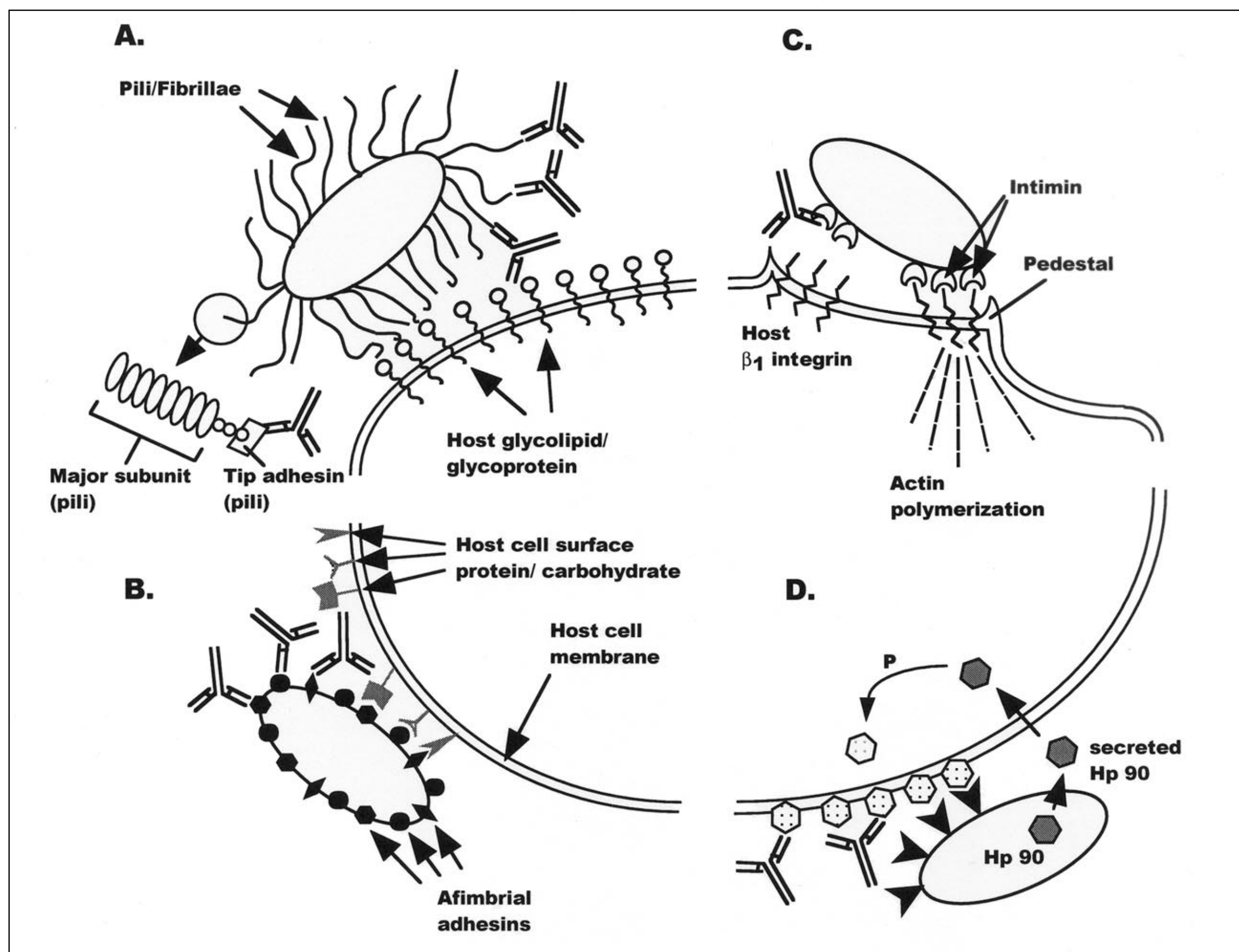

Figure 1. Four mechanisms of bacterial adherence where anti-adhesin vaccines could potentially block colonization and infection. A shows pili or fibrillae protruding from the bacterial surface. These proteinaceous appendages bind to host cell surface molecules, usually carbohydrates, by adhesin proteins located at the distal tip of the pilus/fibrillar organelle. Antibodies targeting the adhesin protein block the bacterial/host interaction. B demonstrates a similar process of bacterial/epithelial cell interactions mediated by afimbrial adhesin proteins. In this case, antibodies directed against the bacterial surface proteins should also block attachment and colonization by impeding the ability of the bacteria to associate with mucosal tissues. C illustrates that some bacteria establish intimate associations with eukaryotic cells by intimin proteins, resulting in cytoskeletal rearrangements, host cell signaling, possible internalization of the bacteria, and in many cases systemic disease. Blocking the intimate association/adherence may also be another strategy to prevent bacterial infections. D shows a novel mechanism whereby bacteria secrete their own receptor protein, which is internalized by the target host cell, phosphorylated, and embedded in the eukaryotic cell as a new receptor for tight binding by the bacterium. Theoretically, blocking the secreted receptor (Hp90) before it is internalized by the host cell could provide another mechanism to block bacterial adherence and infection.

FimH adhesin produced by most *Enterobacteriaceae* (including uropathogenic *E. coli*), are highly conserved proteins (13). This lack of major variation is most likely due to the requirement that all pathogenic strains recognize invariant host receptors. Although minor changes in the adhesin protein have been observed (2% divergence) and correlate with decreased or increased affinity for binding to sugars (14), antibodies against a single FimH protein cross-react with >90% of *E. coli* strains expressing the FimH adhesin and block binding to bladder cells in vitro (15,16). Furthermore, antibodies against FimH from a single isolate protect against in vivo

colonization by >90% of uropathogenic strains in a murine model for cystitis (unpub. obs.). This high degree of antigenic conservation is another reason why adhesins may serve as ideal vaccines.

Aside from mediating colonization of the host, bacterial attachment often results in the up-regulation or expression of many other virulence genes encoding various proteins that allow for invasion of the host (Figure 1C) (17-23). The proteins can mediate tighter associations with epithelial cells, trigger epithelial cell–actin filament rearrangements, and induce changes in host-cell signaling and function. In some cases, the bacteria may also secrete a protein that inserts into mammalian cells and serves as a receptor for its own intimate adherence with the host (Figure 1D) (24). Given that cross-talk between pathogenic bacteria and host cells after microbial attachment may trigger expression of virulence factors leading to local inflammation or invasive disease, vaccines that block bacterial attachment may have multiple advantages.

Many studies have demonstrated the utility of vaccines against bacterial surface components in blocking attachment in vitro as well as in vivo (25-28). However, as understanding of the mechanisms of attachment has evolved and characterization of specific adhesin molecules has been refined, new opportunities have emerged for the development of adhesin-based vaccines.

Bacterial Adhesins: Gram-Negative Organisms

One of the best understood mechanisms of bacterial adherence is attachment mediated by cell surface structures called pili or fimbriae. Pili are long, flexible structures that extend outward from the bacterial surface of many species of bacteria and allow for contact between the bacteria and the host cell. Originally pili were thought to be homopolymeric structures composed of approximately 1,000 copies of a single structural subunit (fimbrin) packed in a helical array. However, for many pili, such as the highly characterized Type 1 and Pap pili expressed on *E. coli* and other *Enterobacteriaceae*, they are heteropolymeric structures with minor tip fibrillae proteins located at the distal end of the organelle (11). The specific interaction with receptor architectures on host cell surfaces is mediated by one of these tip proteins, called adhesins. For example, FimH adhesin mediates attachment to α-D-mannosides (29,30) by type 1 pili, while PapG mediates binding to α-D-gal(1-4)ß-D-Gal-containing receptors on host cells by Pap pili (31,32) (Table 1). The specificity of interaction may be involved in conferring tropism to the bladder and kidney tissues, respectively (33,38). Pilus-associated adhesins have been identified in a number of other bacteria as well (Table 1).

Not all adhesins are associated with pili. *Bordetella pertussis* expresses at least two

Table 1. Adhesins of gram-negative bacteria

Adhesin	Strain	Ligand	Reference
Pili family[a]			Hultgren (33)
PapG	*Escherichia coli*	Gala(1-4)Gal in globoseries of glycolipids	
SfaS	*E. coli*	a-sialyl-2,3-b-galactose	
FimH	*E. coli*	Mannose-oligosaccharides	
HifE	*Haemophilus influenzae*	Sialylyganglioside-GM1	
PrsG	*E. coli*	Gala(1-4)Gal in globoseries of glycolipids	
MrkD	*Klebsiella pneumoniae*	Type V collagen	
FHA	*Bordetella pertussis*	Sulfated sugars on cell-surface glycoconjugates	Brennan (34)
Pertactin	*B. pertussis*	Integrins	Brennan (34)
HMW1/HMW2	*H. influenzae*	Human epithelial cells	St. Geme (35)
Hia	*H. influenzae*	Human conjunctival cells	Barenkamp (36)
Le^b-binding adhesin	*Helicobacter pylori*	Fucosylated Le^b histo-blood group antigens	Ilver (37)

[a]Representative examples from the large family of pilus-associated adhesins.
FHA, filamentous hemagglutinin; HMW, high molecular weight; Hia, *H. influenzae* adhesin

putative adhesins on its surface: filamentous hemagglutinin (FHA) and pertactin (34). FHA is thought to mediate attachment to sulfated sugars on cell-surface glycoconjugates, although it may also have other properties. Pertactin is thought to mediate binding by the Arg-Gly-Asp triplet binding sequence characteristic of integrin-binding proteins, although the role of this binding activity in the pathogenesis of *Bordetella* infections is unclear. Both FHA and pertactin are components in the recently approved acellular pertussis vaccine.

In *Haemophilus influenzae,* two families of nonpilus adhesins have been identified: high-molecular weight adhesion proteins (HMW1 and HMW2) and immunogenic high molecular-weight surface-exposed proteins, the prototypic member of which has been designated Hia for *H. influenzae* adhesin (35). Both families are expressed by nontypable *H. influenzae,* which colonize the respiratory tract and cause such diseases as otitis media, pneumonia, and bronchitis. The HMW proteins, which share homology with the *B. pertussis* FHA protein, mediate specific attachment of *H. influenzae* to different types of human epithelial cells in vitro and have been implicated in directing respiratory tract tropism for these organisms. The Hia protein, in contrast, mediates tight association with human conjunctival cells and is present only in *H. influenzae* strains deficient in HMW1/HMW2 expression (36). Given the dichotomy among nontypable strains expressing either HMW or Hia-like adhesins and the serologic conservation at least among the HMW1 and HMW2 proteins, a vaccine based on a combination of such proteins may be protective against disease caused by most nontypable *H. influenzae*.

Another nonpilus adhesin has been identified and purified from *Helicobacter pylori*. The adhesin, called Le^b-binding adhesin, mediates bacterial adherence to fucosylated Lewis b (Le^b) histoblood group antigens, which are expressed along the mucosal surface of the gastric epithelium (37). The Le^b-binding adhesin may be involved in conferring tropism for stomach epithelium and allowing pathogenic bacteria to establish an ecologic niche within the gastrointestinal tract. In association with other virulence determinants expressed by *H. pylori* in the stomach, the colonization process ultimately results in ulcer formation.

A unique mechanism has been identified by which certain strains of enteropathogenic *E. coli* that cause severe diarrhea target cells for attachment: Enteropathogenic *E. coli* express and insert their own receptor (Hp90) into mammalian cell surfaces, thereby allowing the bacteria to attach and establish intimate contact with the epithelial cells (24) (Figure 1D). Although a bacterially encoded receptor for a cognate adhesin protein (intimin), Hp90 is expressed and secreted by enteropathogenic *E. coli* before colonization; therefore, it may also serve as a target for vaccine development.

Bacterial Adhesins: Gram-Positive Organisms

Some of the most well-characterized colonization factors in gram-positive bacteria—the polypeptides of the antigen I/II family—bind to salivary glycoproteins in a lectinlike interaction (11) and promote adhesion to the tooth surface (Table 2). These proteins include the original AgI/II from *Streptococcus mutans*, also known as SpaP, P1, or PAc, and the *Streptococcus sobrinus* SpaA and PAg proteins (39). *Streptococcus gordonii* expresses two antigen I/II polypeptides, SspA and SspB, products of tandem chromosomal genes (39).

Surface proteins of the antigen I/II family contain alanine-rich repeats, which adopt an α-helical coiled-coil structure, proline rich repeats, and a carboxy-terminal region that includes the gram-positive cell wall anchor motif LPXTG (12,49). Binding activity to salivary glycoprotein has been attributed to both the highly conserved alanine rich repeats (50,51) and the proline-rich repeating sequences (52). In addition to salivary glycoprotein binding activity, SspA has been implicated in coaggregation of *S. gordonii* with Actinomyces (39). Such bacterial coaggregation may be involved in dental plaque formation.

Two other *S. gordonii* proteins, CshA and CshB, have also been implicated in coaggregation with Actinomyces. These adhesins may play a role in adherence of *S. gordonii* to immobilized fibronectin in vitro (52) and in colonization in vivo (40).

Staphylococcus aureus also expresses fibronectin-binding adhesins. Two genes encoding for fibronectin-binding proteins have been identified in *S. aureus*—*fnb*A and *fnb*B (41). Fibronectin binding activity is critical in

Table 2: Adhesins of gram-positive bacteria

Adhesin	Strain	Ligand	Reference
Antigen I/II Family			Demuth (39)
Ag I/II, SpaP, P1, PAc	*Streptococcus mutans*	Salivary glycoprotein	
SspA, SspB	*Streptococcus gordonii*	Salivary glycoprotein, Actinomyces	
SpaA, PAg	*Streptococcus sobrinus*	Salivary glycoprotein	
CshA , CshB	*S. gordonii*	Actinomyces, fibronectin	McNab (40)
FnbA, FnbB	*Staphylococcus aureus*	Fibronectin	Jonsson (41)
SfbI, Protein F	*Streptococcus pyogenes*	Fibronectin	Talay (42) Hanski (43)
LraI gamily			Burnett-Curley et al. (44) Jenkinson and Lamont (12)
FimA	*Streptococcus parasanguis*	Salivary glycoprotein fibrin	
PsaA	*Streptococcus pneumoniae*	Unknown	
ScaA	*S. gordonii*	Actinomyces	
SsaB	*Streptococcus sanguis*	Salivary glycoprotein	
EfaA	*Enterococcus faecalis*	Unknown	
CbpA/SpsA/PbcA/ PspC	*S. pneumoniae*	Cytokine activated epithelial and endothelial cells, IgA	Rosenow (45) Hammerschmidt et al. (46) Briles et al. (47) Smith et al. (48)

pathogenesis because it allows the bacteria to adhere to extracellular matrix components including fibronectin and collagen (53). This can result in cutaneous infections and in life-threatening bacteremia and endocarditis (54).

Binding to fibronectin is also essential for the attachment of *S. pyogenes* to respiratory endothelial cells. *S. pyogenes*, a group A streptococcus, has been implicated in various diseases from skin and throat infections to sepsis and shock (55). This binding activity is mediated by several fibronectin binding proteins. Six different genes encoding proteins with fibronectin binding activity have been identified (12). The most well characterized are two closely related proteins, SfbI (42) and Protein F (43). Both proteins are directly involved in the fibronectin-mediated adherence to epithelial cells.

Another group of extensively studied streptococcal adhesins is the LraI family of proteins. Included in this group are FimA from *S. parasanguis*, PsaA from *S. pneumoniae*, ScaA from *S. gordonii*, ScbA from *S. crista,* SsaB from *S. sanguis*, and *Efa*A from *Enterococcus faecalis* (44,56). These membrane-bound lipoproteins are part of a larger family of ATP-binding cassette metal permeases, involved in the acquisition of manganese (57).

The adhesins FimA and SsaB have high affinity for salivary glycoprotein on tooth surfaces and are involved in colonization of the oral cavity (58). The FimA adhesin for *S. parasanguis* has been localized to the tip of peritrichous surface fimbria on these bacteria (58). FimA is also a major virulence factor of *S. parasanguis* and binds fibrin (44). The ability to bind fibrin has been implicated in the pathogenesis of infective endocarditis.

The *S. pneumoniae* homologue of FimA, PsaA, also has a role in pathogenesis (59,60). While it commonly colonizes the nasopharynx of healthy persons, *S. pneumoniae* is a common pathogen in children and older adults and a leading cause of otitis media, bacterial pneumonia, sepsis, and meningitis. Like its homologues, PsaA may function as an adhesin, according to initial evidence (61). Insertion inactivation of *psaA* resulted in pneumococcal mutants that exhibited reduced adherence to alveolar epithelial cells in vitro (60). However, the PsaA protein may be a permease involved in the regulation of adherence rather than functioning as an adhesin per se (57,61).

Another pneumococcal protein thought to be an adhesin is CbpA (45) (also known as SpsA [46] and PspC [47]), one of a family of choline binding

proteins (CBPs) (i.e., surface proteins noncovalently associated with the phosphocholine on the lipoteichoic acid). CbpA was initially isolated from a mixture of pneumococcal proteins that were able to bind to a choline affinity column. The CBP mix was purified from a strain with an inactivated *psp*A gene (45). The exogenous CBP mix inhibited adherence of pneumococci to type II pneumocytes and endothelial cells in vitro, suggesting that one or more of these proteins may act as adhesins. CbpA was the most abundant component in this mix and was shown to be on the surface of intact pneumococci in an indirect fluorescent-labeling assay. The CbpA protein also reacted strongly with a pool of human convalescent-phase serum. CbpA is thought to mediate adherence of *S. pneumoniae* to sialic acid and lacto-N-neotetraose ligands present on cytokine-activated epithelial and endothelial cells in vitro (45). *cbpA*-defective mutants did not colonize the nasopharynx of infant rats, further supporting its function as an adhesin and potential usefulness as an adhesin-based vaccine (45). In addition, others have shown that this choline binding protein (which they called SpsA) has IgA-binding properties, though the relevance of this function to pneumococcal pathogenesis is unclear (46). Yet another group has demonstrated complement protein C3-binding activity for this protein, which they termed PbcA (48). Whatever its role in pathogenesis, we have demonstrated that the gene for CbpA (SpsA/PbcA/PspC) is highly conserved among the most common pneumococcal isolates, further enhancing its use as a vaccine candidate (62).

Adhesins as Vaccines: FimH as a Paradigm for Adhesin-Based Vaccines To Block Colonization

One of the key aspects of proving the potential efficacy of an adhesin-based vaccine in vivo is the development of an animal model of disease that relies on bacterial colonization of the mucosal epithelium mediated by the specific adhesin of interest. Although seemingly straightforward, testing for protection in small animal models of disease is difficult for various reasons: large doses of in vitro grown bacteria are required to establish mucosal colonization in animals, which does not necessarily mimic the course of infection in humans; specific glycoprotein receptors for some adhesins are lacking in animal mucosal tissues that correspond to the site of colonization in humans; and some bacterial adhesins that are usually expressed as part of a larger structure on the bacterial cell surface (e.g., tip adhesins associated with whole pili) are difficult to purify. Despite these difficulties, adhesin-based vaccines have demonstrated efficacy in protecting against infection, thus proving the usefulness of such molecules as subunit vaccines. Research using the FimH adhesin from *E. coli* provides one such example.

Type 1 pili have long been implicated in bacterial urinary tract infections in humans (63,64). In a murine cystitis model, colonization of the bladder by *E. coli* was shown to depend on growth conditions that favored expression of type 1 pili and in particular required FimH (15,65). Thus, the murine model was a valid small-animal model to prove whether adhesin-based vaccines might block colonization.

Although purifying large amounts of pilus-associated adhesin is difficult (because most adhesins are proteolytically degraded when expressed as independent moieties), Hultgren et al. demonstrated that the FimH adhesin could be stabilized in an active conformation by the periplasmic chaperone FimC, making it possible to purify full-length FimH protein. Vaccination with the FimCH complex elicited long-lasting immune responses to FimH. Sera from mice vaccinated with the FimH vaccine inhibited uropathogenic strains of *E. coli* from binding to human bladder cells in vitro. Vaccination with the FimH adhesin-vaccine reduced in vivo colonization of the bladder mucosa by >99% in the murine cystitis model (15). Furthermore, the FimH vaccine protected against colonization and disease by uropathogenic strains of *E. coli* capable of expressing multiple adhesins. IgG specific for FimH was detected in the urine of protected mice, consistent with our original hypothesis that antibodies directed against an adhesin protein might protect along the mucosal surface. Subsequent studies in a primate model of cystitis have corroborated these findings (Langermann et al., unpub. data). Furthermore, in primate studies we demonstrated a direct correlation between the presence of inhibitory antibodies in secretions and protection against colonization and infection.

While IgG antibodies elicited against adhesins are protective, induction of immune responses along the mucosa can be augmented

by a variety of antigen delivery systems that specifically target mucosa-associated lymphoid tissue and activate the mucosal immune system (4-7,66). These delivery systems include whole-inactivated or live-attenuated bacterial and viral vectors, biodegradable microspheres, liposomes, transgenic plants, and antigens conjugated to or coadministered with the cholera toxin B subunit or attenuated forms of heat labile toxin from *E. coli*. Many of these systems hold promise for future vaccine strategies, but only a few have been tested in humans for safety and adjuvanticity. As these mucosal adjuvants progress further toward approval for use in humans, testing should be done with adhesin antigens to determine if induction of local immune responses enhances the protective efficacy of adhesin-based vaccines as compared with conventional parenteral vaccination. Such studies are under way with the FimH vaccine.

Given the preclinical data with the FimH vaccine, similar efforts should be directed at developing adhesin-based vaccines for a wide range of pathogens. In this regard, additional efforts should also be focused on developing mucosal models of infection. The availability of such models should allow for appropriate screening of adhesin-based vaccines to prevent infections by streptococci, staphylococci, and other pathogens for which vaccine coverage is absent or inadequate.

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Tuberculosis in the Caribbean: Using Spacer Oligonucleotide Typing to Understand Strain Origin and Transmission

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We used direct repeat (DR)-based spacer oligonucleotide typing (spoligotyping) (in association with double-repetitive element–polymerase chain reaction, IS*6110*-restriction fragment length polymorphism [RFLP], and sometimes DR-RFLP and polymorphic GC-rich sequence-RFLP) to detect epidemiologic links and transmission patterns of *Mycobacterium tuberculosis* on Martinique, Guadeloupe, and French Guiana. In more than a third of the 218 strains we typed from this region, clusters and isolates shared genetic identity, which suggests epidemiologic links. However, because of limited epidemiologic information, only 14.2% of the strains could be directly linked. When spoligotyping patterns shared by two or more isolates were pooled with 392 spoligotypes from other parts of the world, new matches were detected, which suggests imported transmission. Persisting foci of endemic disease and increased active transmission due to high population flux and HIV-coinfection may be linked to the recent reemergence of tuberculosis in the Caribbean. We also found that several distinct families of spoligotypes are overrepresented in this region.

Sequencing of the *Mycobacterium tuberculosis* H37Rv genome (1) has facilitated the study of the biodiversity of *M. tuberculosis* around the world (2). We investigated *M. tuberculosis* epidemiology and biodiversity in the Caribbean region, where sequencing data are not available. Caribbean islands possess independent and shared characteristics that justify investigation of both the molecular epidemiology (3) and the evolutionary history of *M. tuberculosis* (4). We used spacer oligonucleotide typing (spoligotyping), double-repetitiveelement (DRE)– polymerase chain reaction (PCR) and IS*6110*-restriction fragment length polymorphism (RFLP) (5-7) to understand the molecular epidemiology and reconstruct the phylogeny of *M. tuberculosis*. The first step was to demonstrate that the chosen molecular methods differentiated isolates with respect to their molecular clonality. The second step was to demonstrate that under proper epidemiologic circumstances, clonality implies active transmission. To eliminate sample bias, we used all data for Guadeloupe during a 3-year period (1994-96) and for Martinique and French Guiana during a 2-year period (1995-96).

M. tuberculosis may have developed through the domestication of cattle, and if so, *M. bovis* was its most probable precursor (8). On the basis of restricted allelic diversity, the speciation of *M. tuberculosis* is estimated to have occurred 15,000 to 20,000 years ago (4). It is thought that the spread of tuberculosis (TB) began in Europe around the middle ages and reached the new world in the 16th century, Africa in the 19th century, and only recently, remote regions such as Papua-New Guinea (mid-20th century) and the Amazon (last quarter of the 20th century) (8). The study of the genetic biodiversity of *M. tuberculosis* might help reconstruct this evolutionary scenario. Previous work from our laboratory showed that a significant proportion of patients had conserved (or ancient) strains of

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tubercle bacilli (6), and analysis based on multiple genetic markers showed genetic relatedness between some clusters of bacilli within Guadeloupe (9).

Demographics and Molecular Typing Methods

Guadeloupe, Martinique, and French Guiana have major demographic differences (Table 1). Guadeloupe and Martinique are densely populated islands in the center of Lesser Antilles; they have similar population sizes (417,000 and 388,000) and areas (1,705 km^2 and 1,100 km^2) and homogeneous populations (93% French nationals). French Guiana is sparsely populated (150,000), its inhabitants scattered throughout a large territory (91,000 km^2). Guadeloupe and Martinique are characterized by an insular population, whereas French Guiana, which borders Surinam and Brazil, is populated by persons of diverse geographic and ethnic origin (many immigrants from South and Central America). The distribution of TB cases among these three French overseas territories reflects their individual demographics. In Guadeloupe and Martinique, 27% and 25% of all TB cases, respectively, were imported, while in French Guiana, 68% of all cases were imported (10). The basic epidemiologic data (incidence, age, sex, nationality, HIV coinfection) for all TB cases reported to health authorities describe a 3-year period (1994-96) (Table 2).

Of 136 TB cases reported in Guadeloupe, 107 had smear- or culture-positive isolates, with culture available in 100 cases; we typed 95 (95%) of 100 isolates. Of 52 cases reported in Martinique, 41 had smear- or culture-positive isolates, and a culture was available in 34 cases. We typed 31 (91%) isolates. Of 124 cases reported in French Guiana, 96 had culture- or smear-positive isolates, with a culture available in 76 cases; we typed 73 (96%) of 76 isolates, along with three that grew in 1995 but came from pathologic specimens received in 1994.

The isolates were first studied by spoligotyping (11), which is based on polymorphism of the DR locus of *M. tuberculosis* (12); spoligotyping results were analyzed by using the Recognizer and Taxotron software (Institut Pasteur, Paris). The 1-Jaccard Index was calculated, and strains were compared by the unweighted pair-group method using arithmetic averages (UPGMA) (13) or by the neighbor-joining method (14). Secondary typing was performed by DRE-PCR (15) or IS*6110*-RFLP (Table 3) (5). To determine clonality between isolates, we used the following algorithm: strains were considered clonal only when a combination

Table 2. Epidemiologic and clinical data for 410 tuberculosis (TB) cases reported to the health authorities from 1994 to 1996 in the French West Indies

	Guadeloupe	Martinique	French Guiana	Total
TB cases	136	89	185	410
Pleuropulmonary cases	115	71	156	342
Total TB incidence[a]	11	8.2	40.6	14.20
Sex ratio	1.7	1.9	1.5	1.65
PDR to INH (%)	4.4	2.2	3.5	3.4
PDR to RIF (%)	1.4	-	-	-
Foreign-born TB patients (%)	24.5	20.3	68	43.2
TB-HIV coinfection (%)[b]	28	19	25	24.6

[a]New cases/100,000 inhabitants, 1994 to 1996.
[b]Total number of known HIV-positive cases among TB patients was 101. However, foreign-born patients represented 50% of all the TB-HIV coinfected patients in Martinique, 60% in Guadeloupe, and 80% in French Guiana. The % shown is a minimal estimation as HIV serology results were available only for 49% of patients in Martinique, 75% in Guadeloupe, and 80% in French Guiana.
PDR, primary drug resistance; INH, isoniazid; RIF, rifampin.

Table 1. Demographic characteristics of Guadeloupe, Martinique, and French Guiana

Data	Guadeloupe	Martinique	French Guiana	Total
Surface area (km^2)	1,705	1,100	91,000	93,805
Population (1990)	387,034	359,579	114,808	861,421
Population (1996)	422,290	388,340	151,780	962,410
Density (inhabitants/km^2)	248	353	2	10
Birth rate/1,000 inhabitants	16.8	14.4	29.2	17.8
AIDS[a]	700	383	561	1,644
HIV-positive (%)[b]	1.4	0.8	1.8	1.3

[a]Cumulated cases on June 30, 1996.
[b]On the basis of 7,087 serologic tests performed in departmental clinics in 1994.

Table 3. Molecular fingerprinting and epidemiologic information from spoligotyping-defined clusters shown in Figure 7.[a]

Spoligo-type no. meth. 1	No. of strains harboring this type	No. of strains typed by IS*6110* RFLP meth.2	DR RFLP meth.3	DRE PCR meth.4	Summary of results obtained by molecular typing methods[b]	Clinical and epidemiologic data, origin, observations
1	2	2	2	2	Different by meth. 2, Beijing IS*6110* patterns	Found in Surinam and Guadeloupe
2	9	9	2	8	No subcluster by PGRS-RFLP: all strains identical by meth. 2 and 4 IS-type J and by PGRS-RFLP	2 patients from hospital A and 2 from B + 2 patients with same surnames
3	3	3	1	3	2/3 strains identical by meth. 2, 3, and 4, IS-type P	Found in Surinam and Guadeloupe, 2 patients from hospital A
5	2	1	1	2	2/2 strains identical by meth. 2 and 4, IS type not yet defined	Found in Martinique and Guadeloupe, no evident epidemiologic link
12	2	2	2	1	One spacer difference with type 14, identical by meth. 2 and 3, IS-type A	Very common pattern, represent both active transmission and reactivation
13	2	2	2	ND	One spacer difference with type 14, identical by meth. 2 and 3, IS-type A	Suspicion of cross-contamination (sampling in the same hospital in 3 days)
14	15	15	13	8	Identical by meth. 2 and 3, identical by method 4 (one band), IS-type A	Very common pattern, represent both active transmission and reactivation
15	2	2	2	2	2/2 strains identical by meth. 2, 3, and 4, IS-type C	Imported cluster (Surinam or Dominican Republic)
17	6	6	1	5	Subclustered by PGRS-RFLP: 17B, 2 strains identical by meth. 2, 3 and 4, IS-type N	17B found in 2 Guadeloupean patients hospitalized in same hospital B
29	5	5	4	5	5/5 strains identical by meth. 2, 3, and 4, IS-type B	3 of 5 Guadeloupean patients hospitalized in same hospital B
30	2	2	2	2	2/2 strains identical by meth. 2, 3, and 4	2 patients from the same part of Guadeloupe
31	2	2	1	1	2/2 strains different by meth. 2	Found in French Guiana and in Guadeloupe, no epidemiologic link
33	2	ND	ND	2	2/2 strains different by meth. 4	Found in French Guiana, no epidemiologic link
34	2	2	1	2	2/2 strains identical by meth. 4, method 2: inconclusive, under investigation	Patients from French Guiana, suspected to be epidemiologically linked
42	3	3	1	3	3/3 strains different by meth. 2 and 4	Found in Guadeloupe (one patient) and French Guiana (2 patients), no link
44	2	2	ND	2	2/2 strains identical by meth. 2 and 4	2 patients from St. Maarten (couple)
45	6	1	ND	4	4/6 strains identical by meth. 4. (2: pending)	5 patients from Martinique, one from Guadeloupe, under investigation
46	3	1	ND	3	2/3 strains identical by meth. 4	Found in a Martinique and a Guadeloupe patient, under investigation
50[c]	29	16	8	20	Subclustered by meth. 2, 4 and PGRS-RFLP, IS-type E (3 pat.) and F (2 pat)	Imported clusters (Haïti), other links under investigation
51	4	3	2	3	2/4 strains identical by meth. 2 and 4 (2: pending)	Imported cluster (Haïti) for 2 patients, other links under investigation
53[c]	29	10	8	20	Subclustered by meth. 2, 4 and PGRS, IS-type K (2 pat.) and T (3 pat.)	Patients from cluster T come from the same ward of hospital A, 1996
61	2	2	1	1	2/2 strains identical by PGRS-RFLP, IS results: under investigation	Found in French Guiana in Guadeloupe, under investigation
63	2	2	1	2	2/2 strains identical by meth. 2 and 4, IS-type R	2 patients from hospital A in Guadeloupe, under investigation
64	1	1	ND	1	***	2 isolates from one single patient
65	2	ND	ND	ND	(Results pending)	Found in French Guiana, under investigation
66	2	2	ND	2	2/2 strains identical by meth. 2 and 4	Found in French Guiana, same surname
67	2	1	ND	2	2/2 strains identical by meth. 2 and PGRS-RFLP	Found in French Guiana, under investigation
68	2	1	ND	ND	(Results pending)	Found in one Martinique patient and in Barbados, under investigation

[a]Of 218 isolates typed, 145 isolates were grouped in 27 distinct spoligo-defined clusters, which were further analyzed by one or more typing methods—IS*6110*-RFLP (meth. 2), DR-RFLP (meth. 3), and DRE-PCR (meth.4), and sometimes PGRS-RFLP when DRE-PCR or IS*6110*-RFLP results were inconclusive or unavailable.
[b]Isolates with matching spoligotypes and matching IS*6110* patterns (meth. 1 and 2) or with matching spoligotypes and matching DRE-PCR patterns (meth. 1 and 4) were considered to make up a cluster of epidemiologically associated strains.
[c] Noninformative spoligotype patterns that lack any discriminating power.
*** Two clinical isolates from a single patient.
RFLP, restriction fragment length polymorphism; DR, direct repeat; DRE, double-repetitive element; PCR, polymerase chain reaction; PGRS, polymorphic GC-rich probe; ND, not done.

of spoligotyping plus IS*6110*-RFLP or DRE-PCR indicated they were identical. Data from DR-RFLP (12) and *Sma*I-RFLP that used a polymorphic GC-rich probe (PGRS) (16) and available epidemiologic information are also included in Table 3.

TB Epidemiology

Epidemiologic data for the three territories have been described in detail (Table 2) (10). The mean incidence of cases per 100,000 inhabitants for the period studied was 11 in Guadeloupe, 8.2 in Martinique, and 40.6 in French Guiana. The sex ratio varied from 1.5 in French Guiana to 1.9 in Martinique. In each territory, the highest proportion of TB cases was in persons more than 65 years old; most were reactivated infections. The highest number of cases was in adults 25 to 44 years old; most were new cases due to active disease transmission and the high rate of TB-HIV coinfections. Pleuropulmonary disease was most common (80% to 85% of cases), and drug resistance was rare; primary resistance to isoniazid was 2.2% to 4.4%; single drug resistance to rifampin was not observed in Martinique and French Guiana and was limited to 1.4% of cases in Guadeloupe. Four cases of multidrug-resistant TB (MDR-TB, defined as resistance to at least isoniazid and rifampin) were diagnosed; these cases of secondary drug-resistance were caused by noncompliance to standard antituberculosis chemotherapy. In addition, the rate of TB-HIV coinfection in these three territories was high (19% of total TB cases in Martinique, 25% in French Guiana, and 28% in Guadeloupe). Coinfected patients were most likely men 25 to 44 years of age and of foreign origin (1 of 2 TB-HIV coinfected patients was a foreign national in Martinique, compared with 6 of 10 in Guadeloupe and 8 of 10 in French Guiana). These figures agree with the epidemiologic data concerning the distribution of patients on the basis of their nationality (Figure 1).

In the last 20 years, TB incidence has decreased considerably in these territories. For example, the incidence of new cases in Guadeloupe, 36 per 100,000 inhabitants in 1975, declined to 10 per 100,000 inhabitants in the late 1980s. If the same trend continued, TB would vanish by 2000; however, this decline has slowed and incidence has remained at 10 to 12 per 100,000 since 1989. This reversal of decline is linked to poor economic conditions, increase in unemployment, increase in drug and alcohol abuse, and persistent immigration from countries with a high incidence of TB and ongoing HIV epidemics.

The Spoligotype Database

To define predominant genotypes and trace the origin of strains and their potential movement, we compared spoligotypes of Caribbean isolates with those of other geographic regions. We built a database of 610 spoligotypes (218 from our own investigation and 392 from other countries) with Excel software. The database contains 167 patterns from Goyal et al. (17); 118 from Kamerbeek et al. (11); 106 from

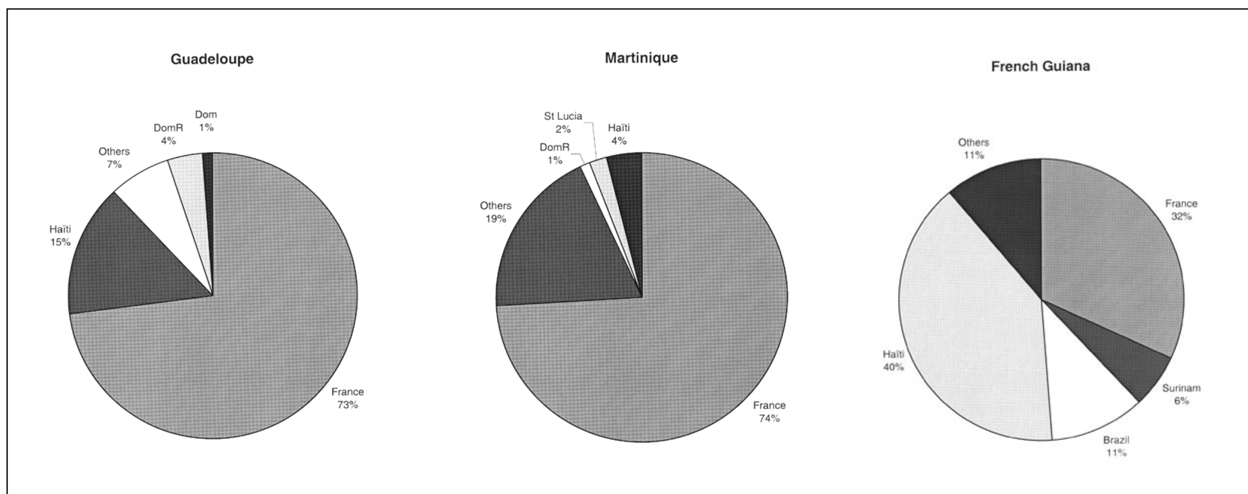

Figure 1. Distribution of tuberculosis cases reported to health authorities in Guadeloupe, Martinique, and French Guiana, 1994-96, by nationality. DomR = Dominican Republic; Dom = Commonwealth of Dominica.

Goguet et al. (18); and a single pattern, named the Manila family, from Douglas et al. (19). In this database, 69 spoligotypes shared by more than two patients in any region of the world were numbered types 1 to 69 (Figure 2): types 1-54 denote 395 spoligotypes that were initially ordered from empty to full squares read from left to right, and types 55 to 69 represent 15 new shared types in chronologic order and correspond to 215 spoligotypes investigated later. The nomenclature is temporary until international guidelines are established. Fourteen shared types might be specific for the Caribbean and bordering Central American regions (patterns 5, 12, 13, 14, 15, 17, 29, 30, 54, 63, 64, 66, 67, 68 [bolded in Figure 2]); 23 shared types were found both in the Caribbean region and in other parts of the world (in regular font in Figure 2). The remaining 32 patterns were not present in the Caribbean (highlighted by an asterisk in Figure 2).

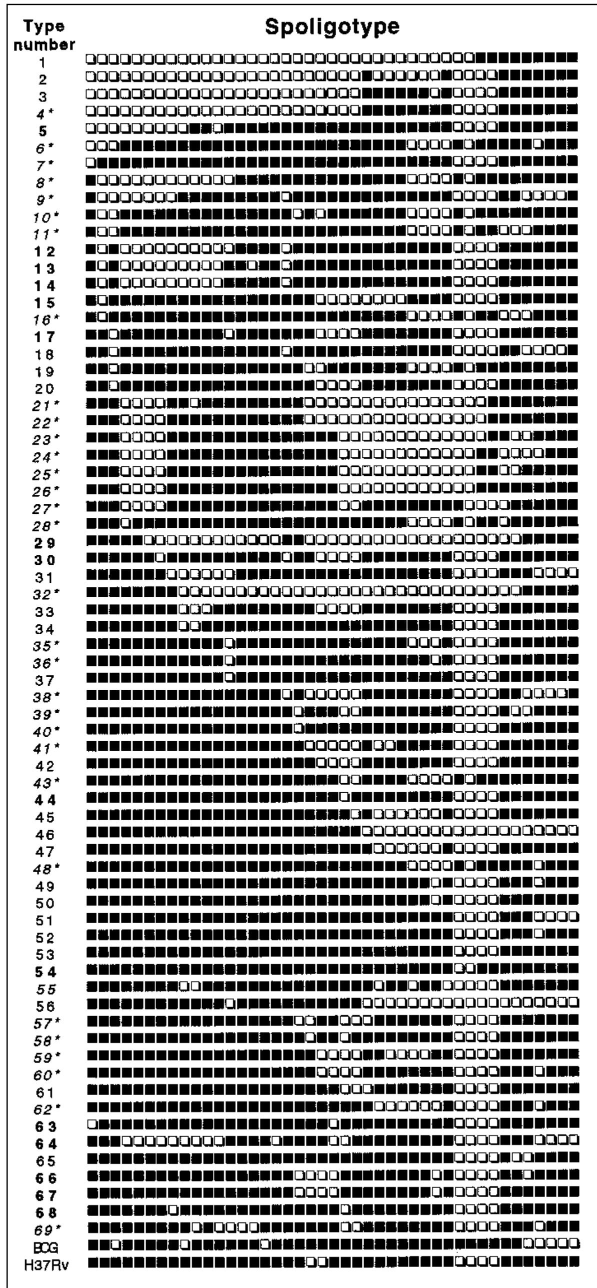

Figure 2. Nomenclature of the spoligotype database (from 1 to 69) based on published spoligotypes (n = 393) and on spoligotypes generated during this investigation (n = 218). The corresponding hybridization patterns for oligonucleotides 1 to 43 (black square, hybridizing; empty square, nonhybridizing) are shown. Type 1 is unique for the Beijing type pattern, whereas type 69 is unique for the Manila type pattern. Bold characters illustrate patterns that have so far been noticed only in Caribbean and neighboring Central American isolates (specific types). Unbolded characters illustrate patterns common to those reported elsewhere (ubiquitous types), whereas italicized characters with an asterisk illustrates patterns not yet found in the Caribbean. BCG and H37Rv spoligotypes are shown as controls.

Population Structure of *M. tuberculosis* in Guadeloupe

Of 95 isolates from Guadeloupe (Figure 3), 34 were a unique spoligotype, and 61 shared 13 patterns (six patterns were shared by only two isolates, and seven patterns were shared by the remaining 49 [3 to 13 isolates per pattern]). Patterns 2 (4 isolates), 14 (13 isolates), 29 (5 isolates), 50 (10 isolates), and 53 (12 isolates) were the major shared patterns from Guadeloupe and made up 72% of clustered isolates. The interpretation of the population structure from Guadeloupe shows the presence of important nodes on the dendrogram. As illustrated in Figure 3 (from bottom to top), three distinct patterns show strain 95076 (designated type 1 in our database and identical to the Beijing type commonly found in Asia [20]), the recently reported pattern 3 (11), and pattern 2 (18), respectively. Above the patterns 1-3 (pattern 1 is not shown) lies the previously undescribed pattern 29, which might be specific to our region. Two very different groups can be seen on the next node (the “lower” and “upper” groups). The “lower” group, which comprises 20 isolates and two shared patterns (17 and 30), shows a stepwise polymorphism. This group contains

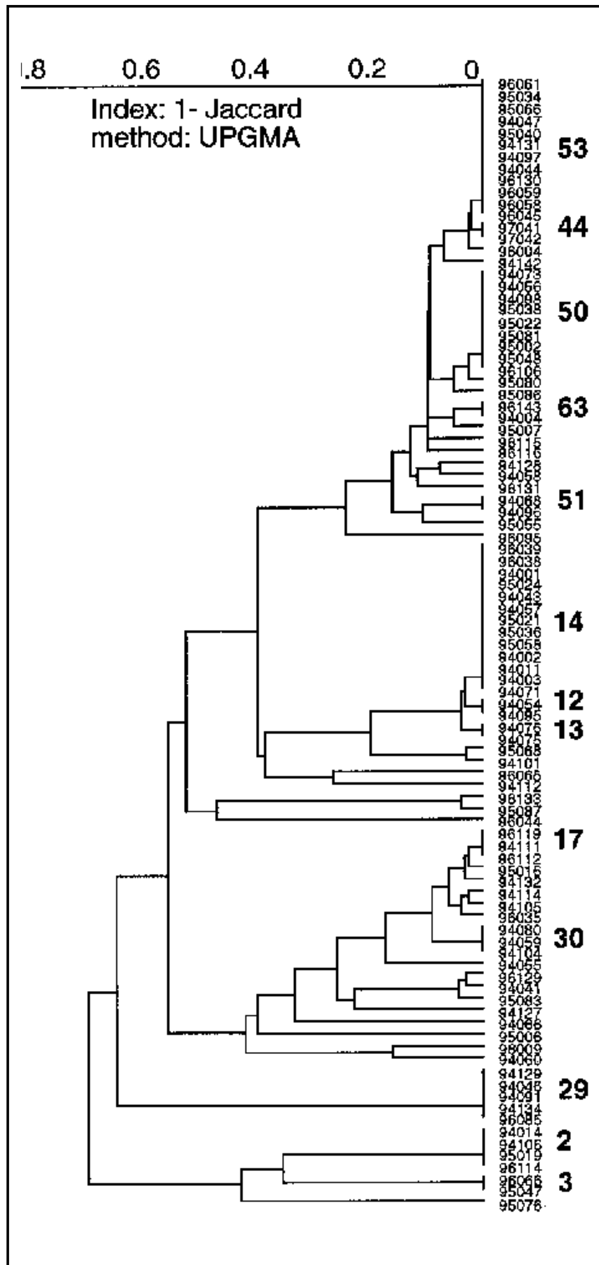

Figure 3. A dendrogram represents spoligotyping results of 95 *Mycobacterium tuberculosis* isolates from Guadeloupe (shared patterns are shown by bold characters). From top to bottom; type 53, ubiquitous; type 44, described by Goguet et al. (two cases) (18); type 54, which does not appear, is found only in isolate 94142, a pattern also found in French Guiana; type 37, which also does not appear, described by Kamerbeek et al. (three cases) (11); type 50, ubiquitous; type 63, specific; type 61, which does not appear is found only in isolate 96131, a pattern also found in Barbados and French Guiana; type 51, described by Goguet et al. (one case) (18); isolate 96095, a *M. bovis* BCG clinical isolate; types 12 to 14, specific types; type 5, which does not appear, is found only in isolate 95068, a pattern also found in Martinique; type 31, which does not appear, is found only in isolate 94112, a pattern also found in French Guiana and already reported by Goguet et al. (two cases) (18); type 46, which does not appear, is found only in isolate 95087, a pattern also found in Martinique and was already reported (18); type 17, specific; type 30, specific; types 45 and 15, which do not appear, are found only for isolates 96129 and 94127, respectively, and share patterns with isolates in Martinique and Surinam; type 29, specific; type 2, previously reported by Goguet et al. (one case) (18); type 3, also found in Surinam, has been described by Kamerbeek et al. (one case) (11); type 1, which does not appear, is found only in 95076, a pattern also found in Surinam, and described (20) as the Beijing type.

many ubiquitous patterns (reported from at least three geographic regions) shared by a variety of isolates from around the world and concerns isolates 95016, 94105, and 94041 of shared types 20, 42, and 47, respectively. The "upper" group, which comprises the remaining 63 isolates and eight shared spoligotypes (types 12, 13, 14, 51, 63, 50, 44, 53), can be further divided into two subgroups: "upper ubiquitous," which lies above *M. bovis* BCG strain 96095 and the "upper specific," which lies below the BCG strain (Figure 3). The "upper specific" subgroup is homogeneous (shared types 12, 13, 14, and strain 95068 of shared type 5) and probably has been present in Guadeloupe for a long time (except in a single isolate with pattern 5 from the neighboring island of Martinique). The "upper ubiquitous" subgroup seems considerably closer to *M. bovis* BCG than do other isolates of *M. tuberculosis* from Guadeloupe.

Population Structure of *M. tuberculosis* in French Guiana

The first population structure of *M. tuberculosis* isolates from French Guiana is shown in Figure 4. Of 76 isolates, 30 had a unique spoligotype. The remaining 46 shared a total of 11 patterns: eight patterns (51, 33, 17, 42, 67, 66, 65, 34) were shared by only two isolates and three major patterns (pattern 2, 4 isolates; pattern 53, 9 isolates; pattern 50, 17 isolates) were shared by the remaining 30 isolates. The three major patterns represented 65% of clustered isolates in French Guiana. Most clusters were common to those found in Guadeloupe, as well as other regions of the world (types 2, 17, 33, 34, 42, 50, 51, 53, 65), except two patterns that have been so far only reported from French Guiana (types 66, 67). No isolate was of the Beijing type, which is unexpected, considering the number of persons of Chinese origin in French Guiana. A high degree of heterogeneity was observed in French Guiana, which is not surprising given the large surface area, high number of immigrants and persons of various ethnic origins, and high TB incidence rate.

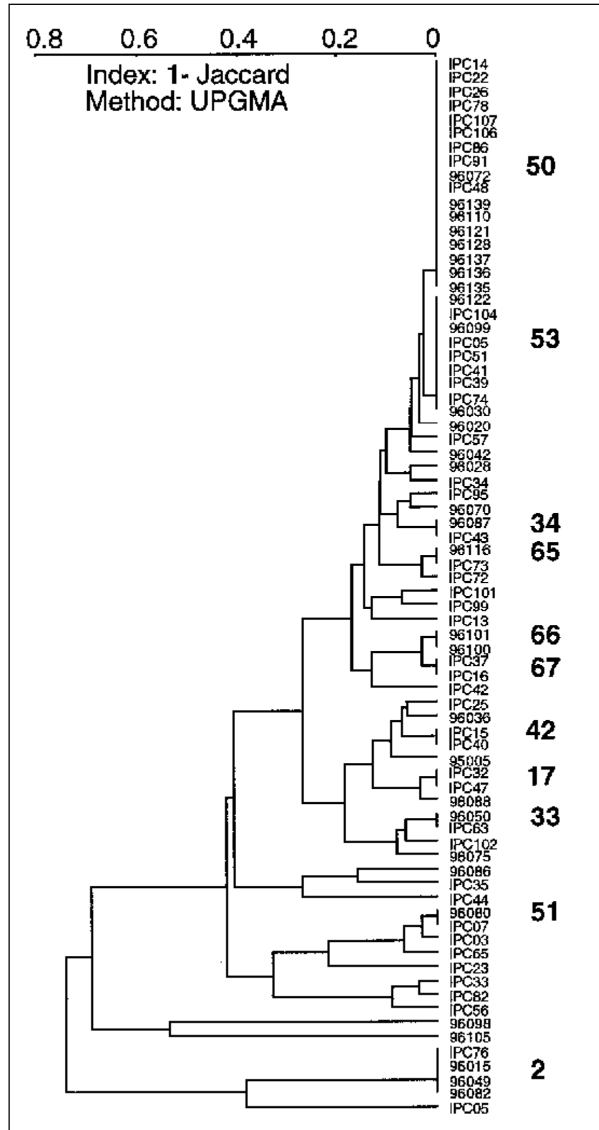

Figure 4. A dendrogram illustrating spoligotyping results of 76 *Mycobacterium tuberculosis* isolates from French Guiana (shared patterns are shown in bold). Top to bottom; patterns 50 and 53, ubiquitous; types 54 and 36, which do not appear, are found only for isolates IPC99 and IPC57, respectively; type 34, a ubiquitous type that was limited to French Guiana in this study; types 66 and 67, specific; type 42, ubiquitous; type 17, specific; type 33, ubiquitous; type 51, ubiquitous; type 31, which does not appear, is found only for isolate IPC23 and is shared with 94112 in Guadeloupe; type 2, shared by four isolates in Guadeloupe.

Population Structure of *M. tuberculosis* in Martinique

Of 31 isolates studied in Martinique (Figure 5), 19 had an unshared spoligotype, and 12

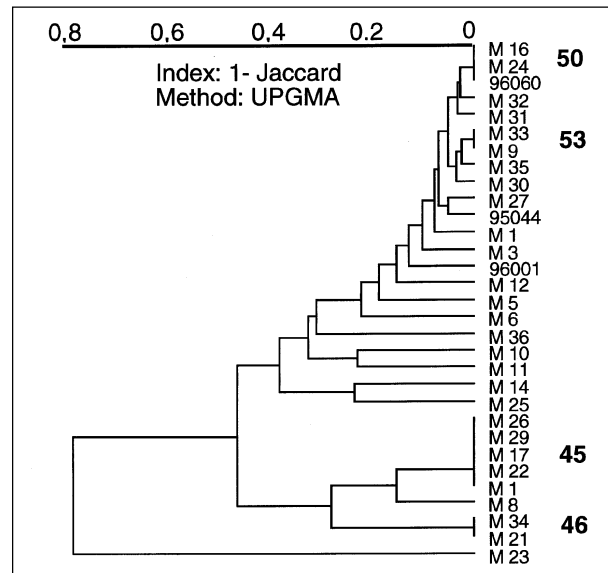

Figure 5. A dendrogram illustrating 31 spoligotyping results of *Mycobacterium tuberculosis* isolates from Martinique. From top to bottom, types 50 and 53, ubiquitous; types 52 and 49 are represented by a single isolate, respectively, M35 and M30, and are ubiquitous; isolates M10 and M7 belong to specific types 17 and 68, respectively found in Guadeloupe and Barbados; M25, belongs to specific type 5 observed in Guadeloupe; type 45, ubiquitous; type 46, ubiquitous; isolate M23 shares type 2, with isolates in Guadeloupe and French Guiana.

shared 4 patterns (pattern 46, 2 isolates; pattern 45, 5 isolates; pattern 53, 2 isolates; pattern 50, 3 isolates). All the clusters in Martinique were also found in Guadeloupe (45, 46, 50, and 53). Pattern 45 is overrepresented in this population, which could suggest active transmission of this clone of tubercle bacilli in Martinique. Other patterns common in Guadeloupe are only poorly represented in Martinique: type 2 (isolate M23), type 5 (isolate M25), and type 17 (isolate M10). Type 5 is specific only to Martinique and Guadeloupe. On the contrary, Martinique shares some patterns with the rest of the world that are not found in Guadeloupe or French Guiana (type 49, isolate M30; type 52, isolate M35). Despite the small sample size of isolates from Martinique, many isolates (clustered or unclustered) share published patterns from the rest of the world (18 of 31 isolates studied).

Population Structure of *M. tuberculosis* in Other Caribbean Regions

We also investigated 16 additional *M. tuberculosis* isolates (12 from Surinam and 4 from Barbados). Although not representative of the population studied, spoligotyping of these isolates allowed detection of some interesting links (Figure 6). Fourteen unique types and one shared type (pattern 53, two isolates) were found. Among the unclustered isolates, strain 94030 from Surinam matched isolate 94127 from Guadeloupe (type 15), strain 94018 from Surinam matched type 19 from Holland (11), and strain 94034 from Surinam matched isolate 95047 (type 3) from Guadeloupe. Furthermore, two strains from Barbados shared patterns with isolates from other geographic areas: Barb 3 to M7 (type 68) from Martinique and strain Barb 1 to the pattern 61 from France (18).

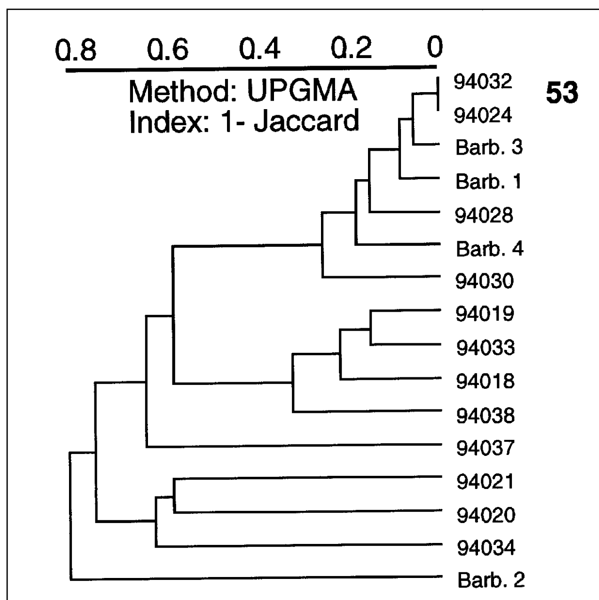

Figure 6. A dendrogram illustrating spoligotyping results of 16 *Mycobacterium tuberculosis* clinical isolates from Barbados and Surinam. From top: type 53, ubiquitous; isolates Barb.3 and 94030 belong to specific types 68 and 15, respectively; isolates 94018, 94020, 94034, and Barb.1 belong to ubiquitous types 19, 1, 3, and 61, respectively.

Predominant Genotypes and Strain Origin and Transmission

The dendrogram representing the global structure of the population studied (Figure 7) shows the potential historical or epidemiologic interregional flux of *M. tuberculosis* between Caribbean and neighboring Central American regions. Of the 218 isolates, 145 (66.5%) had 29 shared types. Twenty-five isolates were not initially grouped but were clustered only when a dendrogram of all 218 isolates was drawn. These interregional links defined eight new types in the database (patterns 1, 5, 15, 31, 54, 61, 64, and 68) for 15 isolates; the remaining 10 isolates enriched patterns already present (Figure 7). These links, made by finding clonal strains in distant geographic territories, do not necessarily define epidemiologic relationships between these strains (21) but are unlikely to be due to independent genetic evolution. The dendrogram is an indirect picture of the common history of TB spread in this part of the world.

For example, spoligotype 2 was recently proposed to have originated in Latin America (22). Our recent investigations favor this hypothesis as we have traced this pattern in all three territories investigated (Martinique [one isolate]), Guadeloupe [four isolates], and French Guiana [four isolates]). Isolates from all nine patients were further investigated by IS*6110*-RFLP, DRE-PCR, and *Sma*I-PGRS typing (23), and the results confirmed the clonality of this specific cluster. Searching for this genotype in other Latin American countries may help elucidate its origin and distribution.

To define the clonality of the isolates and to be in accordance with the current practice for molecular typing of *M. tuberculosis* using spoligotyping as a first-line method (7, 11, 17, 18), we systematically performed a study of a spoligotyping-defined cluster by a second

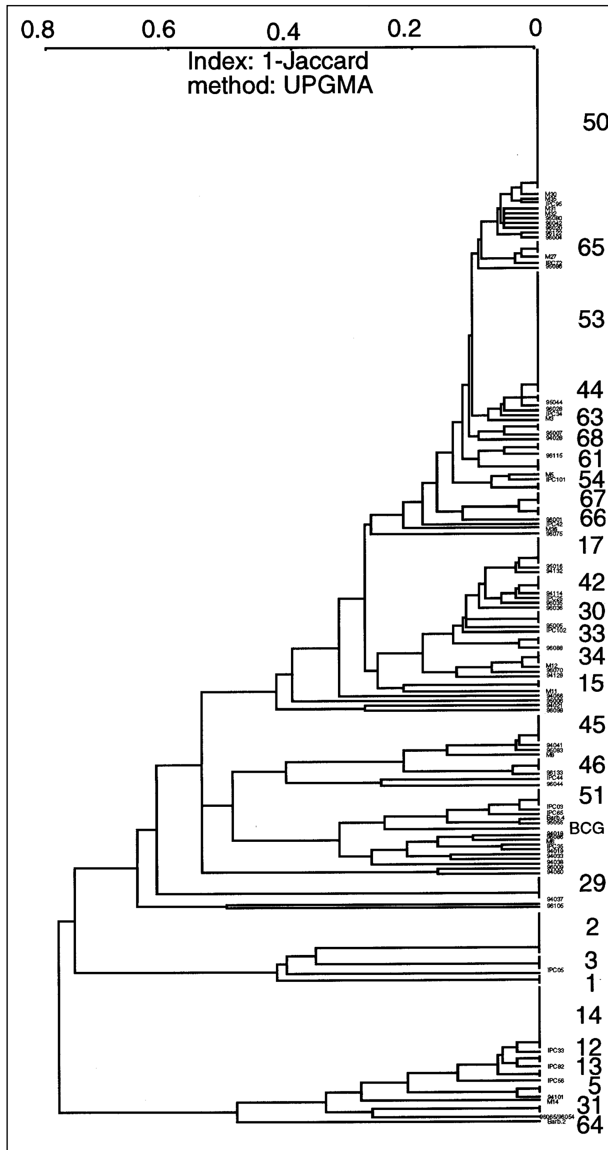

Figure 7. A cumulative dendrogram for the 218 Caribbean isolates of *Mycobacterium tuberculosis*. New types not visible on individual dendrograms can now be observed (types 1, 5, 15, 31, 54, 61, 64, and 68).

method (IS*6110*-RFLP or DRE-PCR). When corroborated by epidemiologic information, the clusters so defined were considered evidence of ongoing active transmission of TB (Table 3). However, in numerous cases, the epidemiologic data were not conclusive, and molecular clonality was not considered direct proof of a link. Thus, despite suspected links in 81 (37%) of 218 of cases on the basis of molecular typing alone, a direct epidemiologic link was demonstrated in 31 (14.2%) of 218 of cases. However, future prospective studies including active case-finding and source tracing may help increase the number of epidemiologically linked cases in our region.

Distribution of Spoligotypes Bearing Geographic Specificities

To reconstruct the evolutionary tree of *M. tuberculosis* on the basis of 610 different spoligotypes (mostly from the United Kingdom, Holland, France, the Caribbean, and the neighboring Central American region), we performed a similarity search (Figure 2). Although the tree may not reflect the full diversity of spoligotypes, it suggests the existence of distinct families of spoligotypes with geographic specificities (Figure 8). Some strain families may be related to specific populations, geographic regions, and the history of TB spread. For example, the Beijing genotype, which is most divergent in the tree shown in Figure 8 (type 1), would have undergone the most extensive genetic evolution since the origin of *M. tuberculosis*. This information is consistent with the mechanism of evolution of the DR locus, which appears to proceed through losses of direct repeats (24,25).

Although in an unrooted tree, the positions of patterns 50 and 53 correlate well with the isolates most widely represented internationally (116 of 610 isolates studied; 54 of 218 isolates from the Caribbean and neighboring Central American region and 62 of 392 published spoligotypes from different parts of the world) and may constitute a candidate root for the interpretation of distances between isolates.

Genetic Evolution of Tubercle Bacilli

The genetic evolution of tubercle bacilli is closely associated with the past and present of its host. Consequently, human migration, population mixing, and other sociodemographic factors have long played an important role in the spread and subsequent evolution of the *M. tuberculosis* genome. The insular model of the Caribbean, in which human migration (hence the introduction of the disease) was estimated to occur only approximately 400 years ago, is particularly interesting for discovering conserved strains of tubercle bacilli and for detecting rare *M. tuberculosis* genotypes. In this context, the DR locus is a unique chromosomal

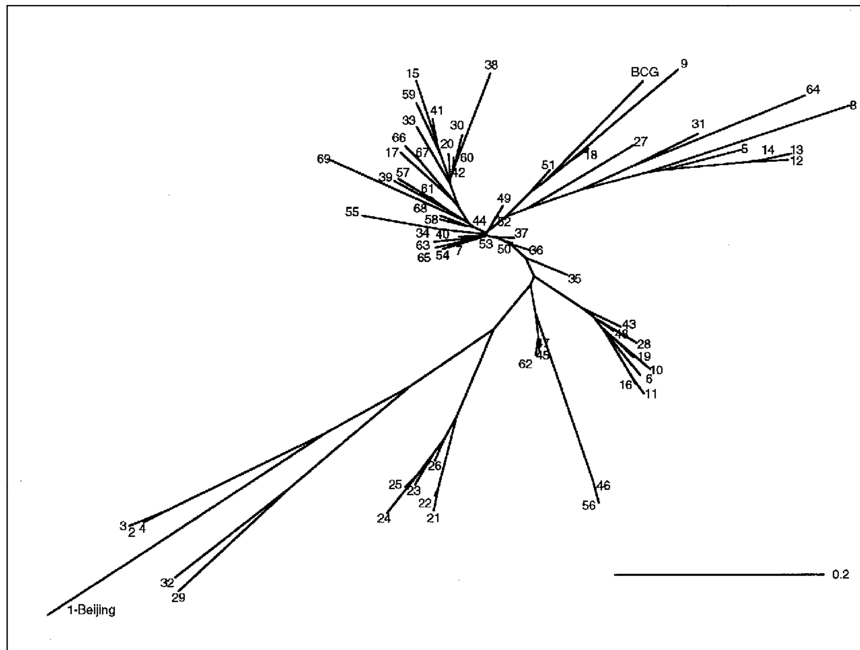

Figure 8. A preliminary phylogenetic tree obtained by the neighbor-joining algorithm on the basis of the 1-Jaccard index (Sj=a/a+c, where a is the number of simultaneously positive characters and c is the number of discrepancies), which is not exhaustive for all existing phylogenetic links. A total of 70 shared spoligotypes were analyzed (69 types shown in Figure 2 and *Mycobacterium bovis* BCG).

region characteristic of the *M. tuberculosis* complex, which shows a high degree of polymorphism that involves homologous recombination and IS-mediated transposition (24). The high degree of internal homology within the DR region of *M. tuberculosis* is likely to favor such genetic rearrangements. Similarly, transposition of IS*6110* is instrumental in generating new subclones of *M. tuberculosis* (26).

Following the principle of Dollo parsimony, which assumes that losses of genes are much more common and likely than independent evolutionary origins (27), the evolutionary process of *M. tuberculosis* can be speculated to have involved the loss of DR repeats. However, the tree in Figure 8 does not perfectly represent all phylogenetic links between isolates as the mechanisms of loss of DR elements (by homologous recombination or replication slippage) could involve simultaneous loss of >1 of the 43 building blocks. The present phylogenetic analysis will be extended to other multiple genetic markers to include variables recently proposed by Sreevatsan et al. (4). However, construction of such trees on the basis of the simultaneous feeding and computer analysis of multiple mycobacterial markers remains cumbersome and constitutes a priority research topic for the studies of *M. tuberculosis* genome evolution.

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Human Rabies Postexposure Prophylaxis during a Raccoon Rabies Epizootic in New York, 1993 and 1994

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We describe the epidemiology of human rabies postexposure prophylaxis (PEP) in four upstate New York counties during the 1st and 2nd year of a raccoon rabies epizootic. We obtained data from records of 1,173 persons whose rabies PEP was reported to local health departments in 1993 and 1994. Mean annual PEP incidence rates were highest in rural counties, in summer, and in patients 10 to 14 and 35 to 44 years of age. PEP given after bites was primarily associated with unvaccinated dogs and cats, but most (70%) was not attributable to bites. Although pet vaccination and stray animal control, which target direct exposure, remain the cornerstones of human rabies prevention, the risk for rabies by the nonbite route (e.g., raccoon saliva on pet dogs' and cats' fur) should also be considered.

Raccoon rabies, present in the southeastern United States since the 1950s, became responsible for an epizootic in the U.S. mid-Atlantic region during the 1970s after raccoons were translocated there for hunting (1). The introduction of the variant of rabies virus associated with raccoons into a rabies-naive raccoon population caused the most intensive animal rabies outbreak on record, in part because of the abundance of raccoons in suburban environments throughout the mid-Atlantic and northeastern metropolitan areas. Raccoon rabies affects approximately one million square kilometers of the eastern United States with a human population of approximately 90 million.

Since the mid-Atlantic raccoon rabies epizootic entered New York State in 1990, the number of rabid animals increased from 54 in pre-epizootic 1989 to 2,746 (89% raccoons) in 1993—the largest number of rabid animals ever reported from any state (2). Despite traditional public health measures for rabies control (e.g., pet vaccination, stray animal control, public education), human rabies postexposure prophylaxis (PEP) rates inevitably increased with the arrival of the epizootic front (3). Preliminary data from New York documented a 4,000% increase in the absolute number of persons receiving PEP, from 81 (1989) to 3,336 (1993) (4). The epidemiologic trends of human PEP in New York State remain largely undescribed.

One of the Healthy People 2000 objectives formulated by the U.S. Public Health Service is to reduce by 50% the need for human rabies PEP by the year 2000 (5). A reduction in the number of PEP cases, which are not reportable, appears unattainable without first defining the numerator, as well as the epidemiologic characteristics of precipitating events leading to suspected rabies exposure and inappropriate treatments.

We describe demographic and animal exposure data associated with human rabies PEP in an area with epizootic raccoon rabies. The epidemiologic description is intended to assist medical practitioners and public health officials in reducing the incidence of human and domestic animal exposure to rabid animals and thus in minimizing the need for PEP in communities affected by the raccoon rabies epizootic.

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The Study

Setting

Four contiguous upstate New York counties (Monroe, Wayne, Cayuga, and Onondaga) were first affected by the raccoon rabies epizootic between December 1992 and June 1993 (Figure 1). Monroe and Onondaga Counties, encompassing the cities of Rochester and Syracuse, are predominantly urban-suburban, with human population densities of 414 per square kilometer and 230 per square kilometer, respectively. Wayne and Cayuga Counties are predominantly rural-suburban, with relatively lower population densities of 57 and 45 people per square kilometer, respectively. The four-county region in western upstate New York comprises 7,090 square kilometers and has an estimated human population of 1,354,377.

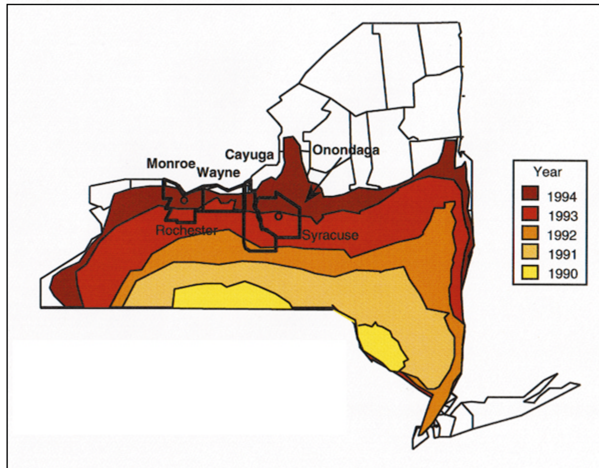

Figure 1. New York State raccoon rabies epizootic progression (1990-94). The raccoon rabies epizootic first affected Monroe, Wayne, Cayuga, and Onondaga Counties between December 1992 and June 1993.

Data Characteristics and Sources

We considered all human rabies PEP cases reported in 1993 and 1994 for the study area. The PEP capture rate was believed high because local health units were responsible for providing funds for any treatment expenses not covered by health insurance, and a completed, rabies report form was required before reimbursement of the local health unit from state funds.

The New York State Sanitary Code requires physicians to report potential human exposure to rabies and PEP administration to county health departments. We abstracted data from these standardized reports and patient records. Data were grouped by patient demographics, animal characteristics, and exposure details. Exposure source was defined as the suspected- or confirmed-rabid animal that directly or indirectly resulted in one or more potential human exposures to rabies. Direct contact exposure consisted of direct contact (e.g., bite, scratch) or contamination of mucous membranes with potentially infectious material from a rabid animal. Indirect contact consisted of contamination from a fomite (e.g., through racoon saliva on a pet's fur with a pet owner's open wounds or mucous membranes).

Analyses

Population figures from the 1990 New York State census were used to calculate the incidence of PEP by county, age, and gender (6). Descriptive analyses of data elements were made through queries of Microsoft Access relational database. Each PEP contributed to the denominator of the analyses. Since multiple PEP cases occurred from exposure to a single animal, data for individual animals were also summarized. Chi-square tests were performed with Epi-Info Version 5 software.

Findings

PEP Incidence

The annual PEP incidence for the study area increased from <1 case per 100,000 residents in pre-epizootic 1992 to 35 cases in 1993 and 52 cases in 1994. Of 1,173 cases of human rabies PEP in the study areas, 474 were reported in 1993 and 699 cases were reported in 1994. The mean annual incidence of PEP was 32 cases per 100,000 for the urban counties (Monroe and Onondaga; 315 residents per square kilometer) and 123 cases per 100,000 for the two rural counties (Wayne and Cayuga; 51 residents per square kilometer).

Season

The number of PEP cases peaked in summer to early autumn (Figure 2). During 1993, the highest number of PEP cases occurred approximately 4 to 6 months (August through November) after the invasion of raccoon rabies during March through June 1993; in 1994, the highest number occurred in summer (June through August).

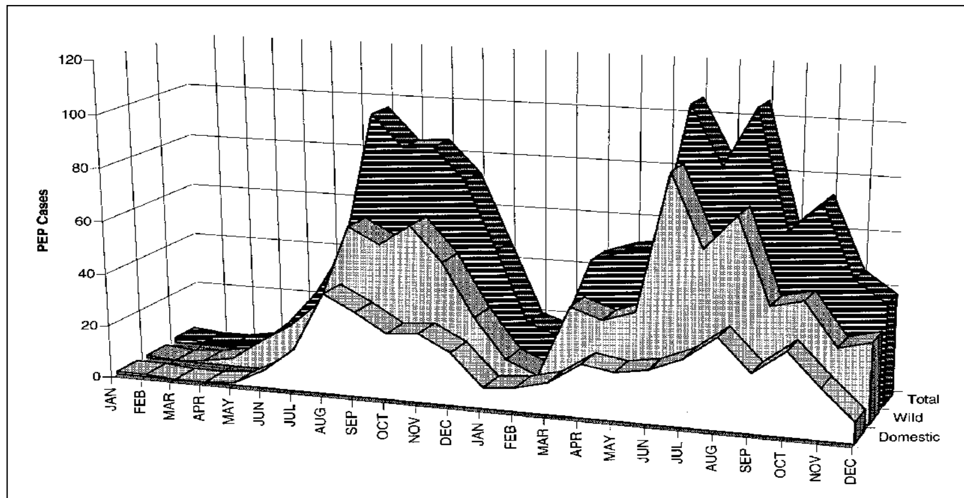

Figure 2. Human rabies postexposure prophylaxis in four New York State counties (Cayuga, Monroe, Onondaga, and Wayne), 1993-1994, by month.

Gender and Age

Gender and age data were available for 100% and 95% of all patients, respectively. Of 1,173 PEP cases, 642 (55%) were administered to male and 531 (45%) to female patients. The mean annual incidence of PEP in male and female patients was 47 and 38 per 100,000, respectively. The PEP rates were highest in persons 10 to 14 years of age (165 per 100,000) and 35 to 44 years of age (113 per 100,000) (Figure 3). The median age was 29 and 31 years for male and female patients, respectively. No significant relationship was observed between gender and age groups for the study area.

Exposure Source Species

Exposure to wild animals accounted for 783 (67%) of all PEP cases (Table 1). Among wildlife, raccoons were by far the leading source of exposure, accounting for 589 (75%) of 779 PEP cases due to wildlife exposure. The other sources of wildlife exposure were bats (54 cases), skunks (35 cases), foxes (28 cases), white-tailed deer (13 cases), woodchucks (12 cases), small rodents (9 cases), sika deer (4 cases), and other wild species (39 cases). Of 390 domestic animal exposures resulting in PEP, 205 were attributed to cats, 165 to dogs, 12 to cattle, 5 to pet rabbits, and 3 to horses. Among PEP cases resulting from exposure to cats and dogs, 66% and 67%, respectively, were initiated after contact with stray animals unavailable for the recommended 10-day confinement and observation to rule out rabies or euthanasia and testing. Dog exposures were disproportionately higher in urban (137 [18%] of 753) than in rural counties (28 [7%] of 420) ($p < 0.001$) (Table 2). In urban areas, dog exposure was primarily due to stray or unowned dogs (95 [69%] of 137). In rural areas, stray or unowned dogs accounted for 11 (39%) of 28 dog exposures ($p < 0.01$). Only one dog (in a rural county) tested posi-

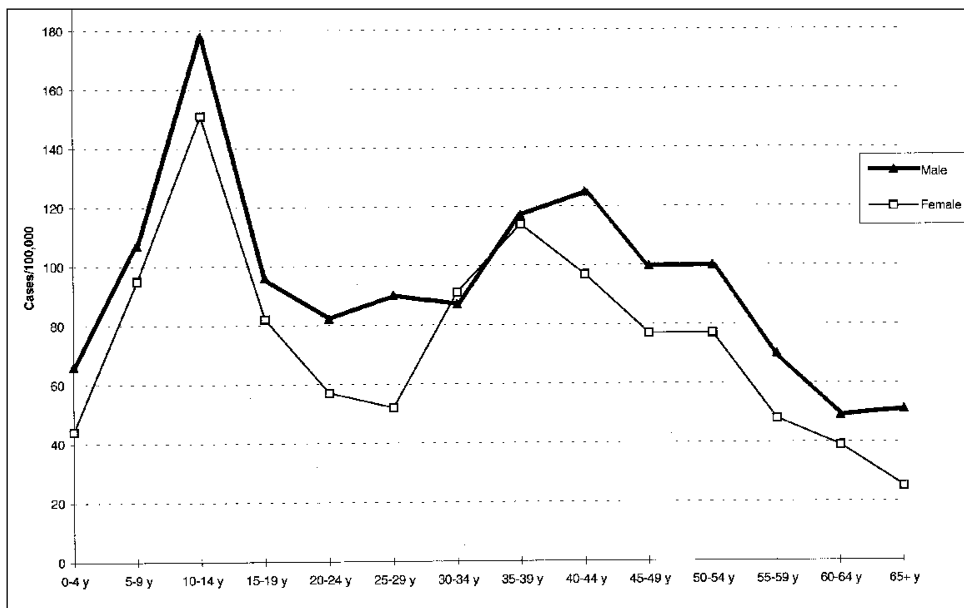

Figure 3. Human rabies postexposure prophylaxis in four New York State counties (Cayuga, Monroe, Onondaga, and Wayne), 1993-94: incidence by gender and age.

Table 1. Human rabies postexposure prophylaxis (PEP), New York State, 1993-94[a]

		Nonbite (N=818)					Overall
	Bite	Direct[b]				Indirect[c]	total
	(N=355)	Scratch	Saliva	NT	Blood	Saliva	PEP
Animal source	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Raccoon	37 (10)	18 (33)	44 (29)	4 (67)	13 (93)	472[d] (79)	589[e] (50)
Bat (all species)	29 (8)	3 (6)	12 (8)	1 (17)	0 (0)	9 (2)	54 (5)
Other wild species	24 (7)	5 (11)	21 (14)	0 (0)	1 (7)	89 (15)	140 (12)
All wild species	90 (25)	26 (47)	77 (51)	5 (84)	14 (100)	570 (96)	783 (67)
Cat	114 (32)	29 (53)	41 (28)	0 (0)	0 (0)	21 (4)	205 (17)
Dog	151 (43)	0 (0)	13 (9)	0 (0)	0 (0)	1 (<1)	165 (14)
Other domestic species	0 (0)	0 (0)	19 (13)	1 (17)	0 (0)	0 (0)	20 (2)
All domestic species	265 (75)	29 (53)	73 (49)	1 (17)	0 (0)	22 (4)	390 (33)
Total	355 (30)	55 (55)	150 (13)	6 (0.5)	14 (1)	592 (51)	1,173 (100)

[a]Data are from Cayuga, Monroe, Onondaga, and Wayne Counties.
[b]Direct contamination of an open wound or mucous membrane with potentially infectious material such as saliva, nervous tissue (NT), or blood (mixed with other body fluids), from a rabies-suspect or known-rabid animal.
[c]No direct contact with a rabid or suspect-rabid animal. Indirect exposure through possible conveyance of saliva on an animal (i.e., pet dog or cat) or inanimate object resulting in contamination of an open wound or mucous membrane.
[d]$p < 0.001$. More people received PEP after indirect exposure to saliva from raccoons than from any other species (472 PEP cases due to indirect contact with 261 raccoons).
[e]Total PEP cases with raccoon as an exposure source (includes one case with no reported route of exposure).

Table 2. Human rabies postexposure prophylaxis (PEP) in New York State, 1993-94: urban and rural settings[a]

Animal source	Urban N (%)	Rural N (%)	All four counties N (%)
Dog[b]	137 (18)	28 (7)	165 (14)
Cat	130 (17)	75 (18)	205 (17)
Other domestic species[c]	5 (<1)	15 (4)	20 (2)
All domestic species	272 (35)	118 (28)	390 (33)
Raccoon	341 (45)	248 (59)	589 (50)
Bat (all species)	41 (5)	13 (1)	54 (5)
Striped skunk	29 (4)	6 (<1)	35 (3)
Fox 19 (3)	9 (2)	28 (2)	
Other wild species[d]	51 (4)	26 (2)	77 (7)
All wild species	481 (65)	302 (72)	783 (67)
Total	753	420	1,173
Rate per 100,000 pop.	32	123	43

[a]Characteristics of human rabies PEP cases reported to the health departments of the two relatively urbanized counties, Onondaga and Monroe, and the two relatively rural counties Cayuga and Wayne, during 1993 and 1994.
[b]$p < 0.00$. Human PEP rates due to dog exposures were significantly higher in urban counties.
[c]Other domestic species include 2 and 3 PEP cases due to cow and horse exposure in the urban counties and 10 and 5 cases due to cow and domestic rabbit exposure in the rural counties, respectively.
[d]Other wild species includes 17, 6, 4, 2, 2, and 1 PEP cases due to an unknown animal type, wild rodent (other than woodchuck), 4 Sika deer (exotic, captive species), opossum, coyote, and mink in the urban counties and 17 and 3 PEP cases due to an unknown animal type and wild rodent (other than woodchuck) in the rural counties, respectively.

tive for rabies in the study area in 1993 and 1994. Of 68 pet cats resulting in human exposure, 61 (90%) were not vaccinated against rabies compared with 14 (24%) of 58 pet dogs ($p < 0.001$).

Type of Exposure

Of 1,173 PEP cases, 355 (30%) resulted from animal bites and 817 (70%) from nonbite encounters (Table 1). A route of exposure not reported in one case involved a raccoon. Suspected contact with animal saliva (148 cases from direct contact and 594 from indirect contact) was responsible for 742 (91%) of 817 PEP cases due to nonbite exposure; contact with nervous tissue (6 PEP cases) or blood (14 PEP cases) accounted for 2% of cases due to nonbite exposure. Fifty-five (7%) of 817 nonbite exposures were attributable to scratches from 23 cats (responsible for 29 PEP cases), 16 raccoons (18 cases), 3 bats (3 cases), 2 wild rodents (2 cases), and 3 other wild animals (3 cases). Of 355 bite exposures, 265 (75%) involved domestic animals (151 due to 150 dogs, 114 due to 108 cats); 90 (25%) involved bites from wild animals—34 raccoons (responsible for 37 PEP cases), 27 bats (29 cases), 9 rodents (9 cases), and 13 other wild species (15 cases).

Mode of Contact

Of 1,173 cases, 594 (51%) occurred because of possible indirect contact with a suspect rabid

animal; 583 (98%) of 594 occurred after suspected exposure to saliva from a rabid (or suspect rabid) animal on the fur of a nonsuspect dog, cat, or other animal. In 9 (2%) of these cases, PEP was administered after suspected exposure by possibly contaminated fomites including door knobs, traps, arrows, a flashlight, and a wire. Possible indirect exposure to dogs with potentially infectious material on their fur resulted in 507 (85%) PEP cases, while suspected indirect exposure by cats resulted in 70 (12%) cases. Other suspected exposure sources were a horse, rabbit, pet duck, chicken, wild bird, captive exotic sika deer, and a person.

Group Exposure

Exposure of one person to a suspect rabid animal precipitated 625 (53%) PEP cases; the remaining 548 (47%) occurred after more than one person was exposed to the same suspected animal (Table 3). Exposure of a single person was more likely associated with a bite ($p < 0.001$), while group exposure (involving two or more persons) was more likely associated with nonbites ($p < 0.001$). Wild animal species accounted for most group exposures—with three exceptions. The largest group exposures (involving 12, 13, and 14 people) were associated with the handling of rabid domestic animals (before diagnosis) by veterinary clinic employees.

Rabies Status

The laboratory diagnosis of rabies in the exposing animal was associated with 540 (46%) of all PEP cases (445 due to wildlife and 95 due to domestic animals). Eighty-nine percent of PEP cases attributed to rabid wildlife involved raccoons. In 88 cases, PEP was initiated after contact with animals eventually proven nonrabid. In 544 cases PEP was administered after contact with animals not tested for rabies. Confirmation of rabies in suspect domestic animals occurred in association with 91 (23%) of 390 PEP cases resulting from exposure to domestic species, including 40 due to 5 pet cats, 23 to 5 stray cats, 13 to 1 pet dog, 5 to a domestic rabbit, 7 to a cow, and 3 to a horse. Conversely, in 88 (8%) of all cases PEP was given after encounters with 81 animals subsequently proven nonrabid (35 due to 33 cats, 32 to 32 dogs, 9 to 8 raccoons, 3 to 3 bats, 5 to 2 skunks, 1 to 1 woodchuck, 1 to 1 squirrel, and 2 to 1 muskrat).

Of 540 cases of PEP associated with animals proven to be rabid, 505 (94%) were due to suspected saliva exposure; 22 (4%) and 13 (2%) involved bites or scratches, respectively. Conversely, 71 (81%) of 88 PEP cases associated with nonrabid animals (i.e., laboratory-confirmed as negative or confined and observed to be healthy) occurred after bite exposures. Of the 544 PEP cases associated with animals of unknown rabies status, 48% were due to bites, 45% to suspected saliva contacts, and 7% to scratches.

Wild animals accounted for 98% of the 690 animals submitted and testing positive for rabies in the study area for 1993-94; 613 were raccoons. If animals testing positive for rabies are used as a surrogate for the true incidence, an

Table 3. Human rabies postexposure prophylaxis (PEP) in New York State, 1993-94: Epidemiologic characteristics[a]

	Group size											
	1		2		3		4		5		6+	
Characteristic	N	%	N	%	N	%	N	%	N	%	N	%
Number	625	53	180	15	84	7	112	10	60	5	112	10
No. of sources	625	79	90	11	28	4	28	4	12	2	13	2
Route of exposure												
Bite[b]	328	52	17	9	4	5	3	3	0	0	3	3
Nonbite	296	47	163	91	80	95	109	97	60	100	109	97
Unknown	1	0.2	0	0	0	0	0	0	0	0	0	0
Source of exposure												
Dog or cat	273	44	16	9	3	4	4	4	10	17	64	57
Other domestic species	2	0.3	4	2	3	4	4	4	5	8	6	5
Raccoon	235	38	124	69	57	68	96	86	35	58	42	38
Bat	39	6	12	7	3	4	0	0	0	0	0	0
Other wild species	76	12	24	13	18	21	8	7	10	17	0	0
Mean age (yr)	33.4		32.5		24.8		22.5		21.8		26.0	

[a]PEP data are from Cayuga, Monroe, Onondaga, and Wayne Counties.
[b]Probability of bite exposure for PEP involving single person vs. group of >1 PEP cases, $p < 0.001$.

approximately 20-fold increase in PEP cases per rabid domestic animal compared with each rabid wild animal, regardless of rural or urban region, is seen (data not shown).

Provoked Exposures

A provoked exposure was characterized by intentional, human-initiated interaction with a suspect rabid animal. Cases resulting from provoked exposure accounted for 392 (33%) of 1,173 of all PEP cases; 248 (63%) involved domestic animals. Most cases resulting from provoked exposure of domestic animals involved cats (162 [65%] of 248) and less frequently, dogs (62 [25%] of 248). Wild animals accounted for 144 (37%) PEP cases from provoked exposure.

Time of PEP Initiation

The interval between exposure to suspect rabid animals and initiation of PEP was 0 to 43 days (median 2 days). Bite exposures were associated with no delay in treatment; nonbite exposures were associated with a 3- to 4-day interval ($p < 0.001$).

PEP Regimen

In 1993 and 1994, postexposure biologic products licensed for use in the United States were rabies vaccine adsorbed, human diploid cell vaccine (Imovax), and human rabies immune globulin (HRIG; Hyperab or Imogam). As recommended by the Advisory Committee on Immunization Practices (ACIP), PEP for the rabies-naive person consists of HRIG (20 IU/kg) on day 0 and five doses of rabies vaccine administered on days 0, 3, 7, 14, and 28 (7). Scheduling information was unavailable for our cases.

Administration of PEP biologic products was recorded as complete in 1,016 (87%) of 1,173 PEP cases. Information regarding completion of the treatment series was not available in 15 cases (1%). Appropriate PEP for preimmunized persons consists of two vaccine doses on days 0 and 3 (7) and was administered to 26 persons, accounting for 2% of all cases. Among preimmunized persons, 17 (65%) of 26 PEP cases occurred after occupational exposures by 11 veterinary staff personnel (including two group exposures to proven rabid cats), four wildlife rehabilitators, one health department employee, and one police officer.

In 54 (5%) instances, PEP was discontinued because of lack of clinical signs in 29 dogs (29 PEP cases) and 23 cats (25 PEP cases) confined for the recommended 10-day observation period. Moreover, 34 (3%) PEP cases were discontinued because of rabies-negative laboratory results in 10 cats (10 PEP cases), 7 raccoons (8 cases), 2 skunks (5 cases), 4 dogs (4 cases), 3 bats (3 cases), 1 muskrat (2 cases), 1 woodchuck (1 case), and 1 squirrel (1 case).

After PEP was initiated, 29 (2%) of 1,173 refused to complete the series; two cited adverse reactions. In nine cases PEP deviated from ACIP recommendations: apparently inadvertent scheduling and administration of six total vaccine doses in four patients and intentional omission of HRIG in the treatment regimen of five patients.

Adverse Effects

The categories available for characterizing adverse effects on the state rabies report form were none, slight, moderate, severe, or unknown. In 596 (51%) of 1,173 PEP cases, no information was recorded. Of 577 responses, 495 (86%) reported no adverse effects resulting from PEP. Adverse effects were characterized as slight by 67 (12%) persons. Moderate adverse reactions including vomiting, nausea, fever, aches, and weakness were reported by 13 (2%) persons. Serious systemic adverse reactions, recorded as anaphylactic shock and serum sickness, occurred in two (0.2%) persons. Both of these patients had received HRIG; PEP was discontinued after one and two vaccine doses in each case.

Conclusions

The most important finding of this study was that in most cases PEP was administered because of suspected nonbite, indirect exposure to animal saliva, a route conventionally thought of nearly negligible risk in rabies transmission (7,8). Because of effective PEP, public health personnel and health-care workers are primarily challenged with the assessment of exposure to rabies, rather than with treatment of human cases of the disease. Assessment of nonbite saliva exposures are particularly time-consuming and should consist of a thorough, but nonleading, history-taking that elicits the probability or confirmation of mucous membrane or nonintact skin contact and a realistic assessment of the

potential presence of infectious saliva on surfaces or pets. Given the invariably fatal outcome of clinical rabies, the tendency may be to administer PEP, even without clear indication of exposure. This tendency may be unwise—not only for economic reasons, but also because, despite their relative innocuity and high potency, modern rabies biologic products, are not risk-free, nor is their supply unlimited.

The first descriptive study of PEP cases associated with the mid-Atlantic raccoon rabies epizootic during 1982-83 (133 patients) also documented that most PEP cases were due to nonbite exposures; however, these principally involved direct exposure to the suspect rabid animal (1).

A 1980-81 nationwide survey of 5,634 PEP cases found an increased risk for occupational and recreational exposure to animals in a rural setting (9). The absolute mean annual PEP rate described in our report of 43 per 100,000 was nearly 10-fold higher than the rate of 4.7 per 100,000 reported in that study. A rate of 66 per 100,000 was reported from two counties (93 people per square kilometer) in New Jersey at the raccoon rabies epizootic front in 1990 (10,11). The incidence of human rabies PEP in New Jersey and this study exceeded by 10- to 20-fold the rates in areas reporting rabies in skunks (12,13), raccoons (14), and mixed wildlife (9,15). The disparity may be partially explained by regional epizootic versus enzootic status of wildlife rabies and subsequent variations in the comparative intensity of disease in wildlife populations, as well as recent increases in both human and animal population densities and their close association in suburban settings (2). The previous PEP studies involved communities in which rabies had been enzootic in terrestrial wildlife for decades (9,12-16). However, the mid-Atlantic raccoon rabies epizootic comprises the emergence of a terrestrial rabies variant into areas that had, for the most part, been free of terrestrial rabies. The exceptions were sporadic cases of spillover from geographically widespread, but low-level, bat rabies into terrestrial animals and occasional incursions of red fox rabies from Canada into New York, Vermont, and other northern states (2,16).

Previous studies of PEP trends in the United States identified bites from dogs and cats as the most common animal encounter, accounting for 65% to 84% of PEP cases (7,9,12-15). By contrast, only 23% of PEP cases in this study were associated with dog or cat bites. In view of current epidemiologic trends in canine rabies-free areas of the United States, if a biting dog appears clinically normal and can be confined and observed for signs of rabies, the decision to administer PEP may be based on suggestive clinical signs and a prompt diagnostic evaluation that confirms rabies rather than on presumptively initiating PEP. Given that cats are now the leading rabid domestic animal in the United States (17), and more specifically that 12 of the 13 domestic species confirmed rabid from the four-county study area during 1993 and 1994 were cats, rabies vaccinations for cats should become more prevalent. Among exposures to owned domestic pets that resulted in human PEP, 9% of cats (versus 76% of dogs) were vaccinated against rabies. Moreover, most of the encounters with dogs that precipitated PEP in urban counties involved bites from stray dogs, indicating the need for enhanced programs for urban dog control.

The economic impact of a new terrestrial rabies variant is substantial (2,18). In 1994, the New York State Department of Health increased its reimbursement to local health units for mandated rabies control activities from $75,000 to $1,080,000 to assist in the expense associated with human rabies PEP, animal rabies testing (11,896 specimens in 1993), and pet immunization clinics (114 in 1993) (4). Local health units in New York State provide funds for treatment expenses not covered by health insurance. With the cost of rabies biologic products alone exceeding $1,500 per treatment series, an exponential increase in the incidence of PEP, as documented in this study, taxes the public health infrastructure. Moreover, unlike red fox rabies, which periodically reinvades northeastern New York from adjoining areas of Canada and Vermont but then dies out, raccoon rabies is expected to persist in affected areas of New York State, as it has in the southeastern United States for the past 5 decades and in the mid-Atlantic and northeastern states more recently (17).

Control of canine rabies in the United States and other industrialized countries was achieved by eliminating the susceptible reservoir population (through stray dog control and mandatory vaccination) (16). Applications of this concept to

wildlife is problematic because of the difficulty in capturing wild animals for vaccination or for applying lethal measures. Population reduction alone is not sufficient to control or eliminate terrestrial wildlife rabies variants over large geographic areas (16). An emerging alternative is oral rabies vaccination of free-ranging reservoir populations, although current methods are still in their infancy and the cost-benefit of such interventions warrants further investigation (10).

During the enzootic raccoon rabies in the southeastern states since the early 1950s or the current mid-Atlantic/northeastern United States epizootic, this variant has not been known to cause human rabies deaths. Yet its potential lethality for humans is supported by ample spillover into other wild animal species (predominantly skunks, but also red foxes, bobcats, and woodchucks) and into domestic animals (predominantly cats, but also dogs, cows, horses, goats, and rabbits). Substantial amounts of infectious rabies virus have been identified in the salivary glands of rabid raccoons (19). No biologic or epidemiologic data suggest unique attenuation or change in virulence of this particular rabies variant that would account for a lack of identified human deaths. Instead, epidemiologic data regarding PEP after suspected exposure to raccoon rabies indicate that PEP frequently is administered even when no exposure has been identified. Also, a bite, scratch, or other exposure, such as gross contamination of an open wound or mucous membrane with moist, infectious material from a small carnivore such as the raccoon, would unlikely be unrecognized or ignored. The apparent liberal administration of effective PEP after known bites, scratches, and other suspected exposures from rabid raccoons may have resulted in complete prevention of human deaths due to this variant of rabies virus associated with raccoons.

Although the Healthy People 2000 goal to reduce PEP is worthwhile, better understanding of the circumstances leading to human exposure and formulating ways to reduce exposure is required to meet this objective. Until then, it will be particularly difficult to reduce PEP during an ever-expanding raccoon rabies epizootic.

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Factory Outbreak of *Escherichia coli* O157:H7 Infection in Japan

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To determine the cause of a July 1996 outbreak of *Escherichia coli* O157:H7 among factory workers in Kyoto, Japan, we conducted cohort and case-control studies. Eating radish sprout salad during lunch at the factory cafeteria had been linked to illness. The sprouts were traced to four growers in Japan; one had been associated with an outbreak of *E. coli* O157:H7 among 6,000 schoolchildren in Sakai earlier in July.

During May through August 1996, approximately 10,000 cases of *Escherichia coli* O157:H7 infection associated with at least 14 separate clusters were reported in Japan (1,2). Most cases occurred in school-age children. One cluster was a large outbreak in Sakai City, Osaka Prefecture, involving more than 6,000 primary school children. The outbreak started on July 13, 1996, and an investigation suggested that radish sprouts were the most likely cause (2,3).

An outbreak also occurred in a factory in Kyoto, approximately 50 km from Sakai City. On July 17, 1996, a 24-year-old male factory worker went to a local clinic with diarrhea. The next day, a second worker came to the clinic with diarrhea. Bloody diarrhea and hemolytic uremic syndrome (HUS) subsequently developed in both patients, and stool cultures from each yielded *E. coli* O157:H7. On July 21, a third worker died of HUS-associated encephalopathy; his stool culture later yielded *E. coli* O157:H7. All three workers had recently eaten meals at the factory cafeteria. To identify a possible food vehicle, we conducted an epidemiologic investigation.

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The Study

On July 19, factory officials requested that ill workers report to factory health-care workers any symptoms from the beginning of July. Stool samples from workers with diarrhea were cultured for *E. coli* O157:H7 and other bacterial pathogens (e.g., *Salmonella* and *Shigella*). Surveillance continued until the end of July. A culture-confirmed case was defined as a stool culture yielding *E. coli* O157:H7 from a factory worker who had onset of diarrhea during July 15 to 22, 1996. A clinical case was defined as diarrhea with one or more loose stools per day with onset during July 15 to 22, 1996.

During their shifts, workers could eat any of the meals served at the factory cafeteria, which was operated by an outside company. Data on the date, time, and type of meal purchased at the cafeteria were routinely recorded by computer, and the cost of meals was deducted from employees' salaries. Workers could not pay by cash. We analyzed these data for visits to the cafeteria during July 8 to 14 (2 to 8 days before the date of symptom onset for the first case of culture-confirmed infection). All factory workers were included in analyses implicating a specific date of eating at the cafeteria. Only factory workers who purchased food at the cafeteria on a particular day were included in analyses of a particular meal for that day.

Two set lunches with prespecified food items were served for the same price in the cafeteria each day. Because the lunches could not be distinguished by computer records, a self-administered questionnaire was completed during September 24 to 27, 1996, by the 47 workers who had reported diarrhea and 300 randomly selected workers who had eaten at the factory cafeteria on the suspected exposure days (July 11 or 12) and had not reported diarrhea in July. A computer record of the meals purchased by each of the workers was included with each questionnaire to assist with recall.

Published methods were used to calculate odds ratios (ORs), 95% confidence intervals (CIs), and p values (4). P values were calculated by a chi-square test: p values of <0.05 were considered significant, and those of 0.05 to 0.09 were considered borderline significant. Multivariate conditional logistic regression analysis was conducted with Statistical Analysis System (SAS) software (SAS Institute, Cary, North Carolina, USA, 1990).

After reports of the first three cases, fecal samples from ill factory workers were cultured in sorbitol indole pyruvic acid bile salts agar (SIB) medium at 35° C to 37° C for 18 to 24 hours at the Kyoto City Institute of Health and Environmental Sciences. To differentiate *E. coli* O157:H7 from other bacteria, colonies were examined on triple sugar iron agar, sulfide indole motility medium, lysin indole motility semisolid agar, Voges-Proskauer semisolid medium, and Simon's citrate agar. Cultures that conformed to the biochemical pattern of *E. coli* O157:H7 were then serotyped. The presence of Shiga toxin 1 or 2 was confirmed by reversed passive latex agglutination and polymerase chain reaction (PCR). Stored food samples were homogenized, and a portion was cultured in modified *E. coli* broth before culturing in SIB medium. To differentiate strains of *E. coli* O157:H7, pulsed-field gel electrophoresis (PFGE) and random amplified polymorphic DNA-PCR (RAPD) assays were performed as previously described (5,6).

On July 18, the regional public health center examined the factory cafeteria kitchen facilities for deficiencies. All 25 food handlers were asked questions regarding abdominal symptoms and provided stool samples for bacteriologic testing. All food served was traced to the distributor and grower as far back as possible.

Findings

Of the 3,155 employees of the factory, 74 reported gastrointestinal symptoms in July; stool samples were obtained from these workers. Illness in 47 persons met the case definition: 42 cases were clinically defined, and 5 were culture-confirmed. The peak date of symptom onset was July 17 (Figure). Six workers had only abdominal pain, fever, or general fatigue, and 21 had onset of diarrhea outside the defined period. HUS developed in three workers with culture-confirmed *E. coli* O157:H7 infection; two fully recovered; one died. One clinical case-patient and four culture-confirmed case-patients had bloody diarrhea. The proportion of cases with bloody diarrhea was 11% among all patients. The median age of case-patients was 30 years (18 to 61). Of the 47 case-patients, 45 (96%) (including all culture-confirmed cases) had eaten at the factory cafeteria during July 8 to 14. Of the 47 case-patients, 39 (83%) were male, and eight (17%) were female. No information on sex and age of the other factory workers was available.

Because the five workers with culture-confirmed *E. coli* O157:H7 infection had no common eating exposure except the factory cafeteria, we first analyzed the association between illness and date of eating at the cafeteria. Eating in the cafeteria any day during July 8 to 13 was associated with illness by univariate analysis. On multivariate logistic regression analysis, this association was significant or borderline significant only for July 11, 12, and 13. The ORs (95% CI, p value) of eating on July 11, 12, and 13 were 2.58 (0.91 to 7.36, 0.08),

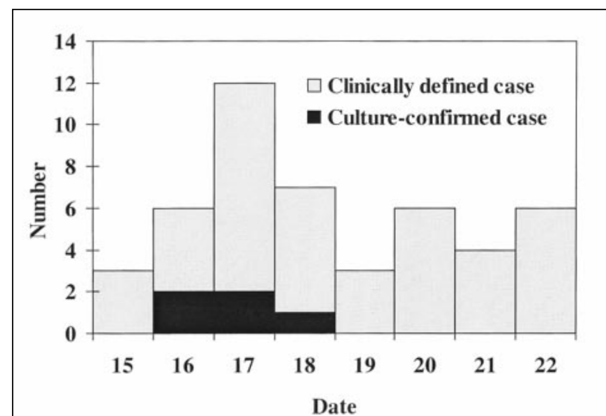

Figure. *Escherichia coli* O157:H7 infection by date of symptom onset, July 15-21, 1996.

2.84 (1.02 to 7.94, 0.05), and 3.19 (1.03 to 9.86, 0.04), respectively. Because 81% of the patients ate in the cafeteria on July 11 and 12 compared with 23% on July 13, July 11 and 12 were considered the most likely days of exposure. On multivariate analysis of the six meal times on July 11 and 12, only eating lunch in the cafeteria on July 11 was associated with illness. The rate of diarrhea for 1,134 workers who ate lunch on July 11 was 3.0%, compared with 0.6% for 2,021 workers who did not (OR = 3.04, 95% CI = 1.08, p = 0.04).

Of the 47 case-patients, 44 (94%) responded to the questionnaire. In 31 patients who answered the question regarding symptoms, diarrhea lasted for a median of 3 days (1 to 10 days), and four (13%) reported bloody diarrhea. Of 300 potential controls randomly selected from factory workers who ate in the cafeteria on July 11 or 12, 291 (97%) responded to the questionnaire. Among the respondents, 16 (5%) reported gastrointestinal symptoms in July (of these, four [25%] had diarrhea during July 15-22, but none reported bloody diarrhea), and three did not respond to the question regarding symptoms; 272 respondents who reported no illness were adopted as controls. The median age was similar for case-patients (30 years [18 to 61 years]) and controls (32 years [20 to 65 years]). Eighty percent of case-patients and 83% of controls were male.

Among the participants, 29 patients and 164 controls responded that they clearly remembered if they had eaten the radish sprout salad. Seventeen (59%) of 29 patients and 64 (39%) of 164 controls reported eating radish sprout salad (OR = 2.21, 95% CI = 0.99 to 4.94, p = 0.08) (Table). No other food item served on July 11 or 12 was eaten by >50% of patients and had a higher odds ratio than radish sprout salad. Among the five patients with culture-confirmed infection, computer records indicated that four patients, including the one who died, ate radish sprouts. Radish sprout salad (consisting of radish sprouts, mayonnaise, cauliflower, and fish paste) was served with both lunches the cafeteria served on July 11, the only time sprouts were served during July 8 to 14.

All five patient isolates of *E. coli* O157:H7 produced Shiga toxins 1 and 2. *E. coli* O157:H7 was not detected in any of the frozen food samples (including radish sprout salad) leftover from cafeteria meals during July 11 to 15. Both PFGE and RAPD patterns of the *E. coli* O157:H7 isolates from this outbreak and the outbreak in Sakai City during the same time were indistinguishable (1-3,7).

Examination of the factory cafeteria kitchen facilities on July 18 by the regional public health center found no deficiencies. One female food handler had diarrhea with onset July 17, but *E. coli* O157:H7 was not cultured from her stool specimen or from specimens of any of the other food handlers.

The radish sprouts served at the cafeteria on July 11 were supplied by a single distributor that received the sprouts from four growers, one of whom also supplied the radish sprouts suspected as the source of *E. coli* O157:H7 infections in the Sakai City school outbreak. Radish sprouts used at the primary schools in Sakai City and at the factory cafeteria had been shipped by the grower on July 9 (3); however, the sprouts used at the factory cafeteria had been purchased along with radish sprouts from different growers.

Conclusions

Our data indicate that the outbreak of *E. coli* O157:H7 infection among Kyoto factory workers was most likely caused by contaminated radish sprouts: the factory outbreak began during the week following the Sakai City outbreak; the factory used radish sprouts from the same grower; they were shipped on the same day as those served to school children in the Sakai City outbreak; and isolates from both outbreaks had indistinguishable PFGE and RAPD patterns (1-3,5,7). The PFGE patterns of earlier outbreaks in Okayama Prefecture (Oku-cho), Gifu Prefecture, Hiroshima Prefecture, Aichi

Table. Factory cafeteria foods associated with illness, July 11, 1996, Kyoto, Japan

Food	Case-patients exposed/total(%)	Controls exposed/total (%)	Odds ratio[a] (95% CI)	p value
Radish sprout salad	17/29(58.6)	64/164(39.0)	2.21(0.99-4.94)	0.08
Boiled beef with soy sauce	8/28(28.6)	24/152(15.8)	2.13(0.84-5.40)	0.18
Scrambled eggs	10/28(35.7)	31/150(20.7)	2.11(0.89-5.04)	0.18

[a]Odds ratio>2.00; CI: confidence interval.

Prefecture, and Okayama Prefecture (Niimi City) were indistinguishable from each other and different from the PFGE patterns of isolates from the outbreaks in Sakai City and the Kyoto factory (1-3,5). *E. coli* O157:H7 was not isolated from radish sprouts; however, the process of freezing sprouts or pooling them with other food items may have decreased the number of organisms to an undetectable level.

Although radish sprouts had never been linked to *E. coli* O157:H7 infection, they are plausible vehicles. Most outbreaks of *E. coli* O157:H7 infections have been linked to ground beef (8), but other items, including unrefrigerated sandwiches (9), apple cider (10), mayonnaise (11), cantaloupe (12), lettuce (13), and alfalfa sprouts (14,15) have been implicated. In addition, some sprout types, including alfalfa sprouts (16) and mung bean sprouts (17), have been linked to *Salmonella* outbreaks. In 1997, *E. coli* O157:H7 was isolated from radish sprouts collected from two different outbreaks of *E. coli* O157:H7 infections in Japan (1).

Three cases of HUS (6%) among 47 cases of clinically or laboratory-defined cases of *E. coli* O157:H7 infection in the factory outbreak is comparable to rates described in other outbreaks (18,19). The proportion of workers reporting bloody diarrhea was low, possibly because infection with *E. coli* O157:H7 follows a more benign course in adults than in children (20) or because the amount of bacterial contamination was low.

Several reasons might explain the small proportion of workers who ate lunch on July 11 and reported illness. First, some ill workers might not have informed the factory health-care personnel about gastrointestinal symptoms for fear of decreasing their chance for future promotion. This seems plausible because 16 (5%) of 291 potential controls in the case-control study mentioned unreported gastrointestinal symptoms. If this percentage of underreporting occurred for the 3,155 workers in the factory, an additional 173 infections may have been missed. Second, the pathogens might have been diluted because only a part of the radish sprout shipment was contaminated. The latter hypothesis is supported by the fact that four growers, including the one implicated in the Sakai City outbreak, supplied the radish sprouts eaten at the factory cafeteria on July 11. Third, the contamination of radish sprouts may have been reduced by washing. Although the association between eating radish sprout salad and illness among workers who ate lunch on July 11 was of borderline significance, it was the only item associated with illness that was consumed by more than 50% of case-patients. Moreover, the next lowest p value was 0.18, far from that of radish sprout salad.

The radish sprout salad contained other food items; therefore, the individual risk for each food item could not be ascertained. However, radish sprouts and mayonnaise were the only uncooked ingredients. Although mayonnaise is a possible vehicle, no reports implicated it in other outbreaks in Japan in 1996 (1).

Recall bias could have occurred in the case-control study because workers were asked about meals they had eaten 8 weeks earlier. Providing with the questionnaire a printout of food items purchased on July 11 and 12 may have assisted recall. In addition, as a result of the outbreak, the cafeteria stopped serving food on July 19 and had not resumed service at the time of the case-control study. This may have assisted respondents in remembering what food items they had eaten during the last week of dining in the cafeteria.

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First Case of Yellow Fever in French Guiana since 1902

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The first case of yellow fever in French Guiana since 1902 was reported in March 1998. The yellow fever virus genome was detected in postmortem liver biopsies by seminested polymerase chain reaction. Sequence analysis showed that this strain was most closely related to strains from Brazil and Ecuador.

Yellow fever (YF) is a serious public health problem in many tropical countries in Africa and South America. In South America, most infections are sporadic, affecting unvaccinated persons who enter the forest; monkeys are the primary reservoirs, and *Haemagoggus* sp. are the vectors. French Guiana, located between Brazil and Surinam in the Amazonian forest, has had many epidemics of YF. However, no case has been reported in French Guiana since 1902, although a serologic survey in 1951 found circulating virus; of 430 persons younger than 50 years of age (who had not been affected by the 1902 epidemic), 9% of those living in the coastal area and 29% of those living inland had significant titers of neutralizing YF antibodies (1). A study conducted at the same time in Surinam showed even higher rates of YF seropositivity in persons who had not been exposed to previous epidemics and confirmed that YF viruses were circulating in the region (2). In French Guiana, YF immunization became compulsory in 1967.

Case Report

In March 1998, an Amerindian woman living in a forest area on the Maroni River was admitted to the health center in Maripasoula, French Guiana, with fever, headache, abdominal pain, vomiting, and diarrhea. She was treated for malaria because of a *Plasmodium falciparum*–positive blood smear. Two days later, the patient's fever increased (40.2°C), she became jaundiced, and she was evacuated to the intensive care unit (ICU) at Cayenne Hospital with multiple visceral failure: shock syndrome, renal failure (blood urea level 32 mmol/l, creatinine level 656 µmol/l), and liver failure (total bilirubin level 314 µmol/l, alanine aminotransferase 2048 IU/l, aspartate amino transferase 6256 IU/l, prothrombin level 23%). No hemorrhages were noted. Despite symptomatic treatment, the condition of the patient deteriorated rapidly, and she died a few hours after admission to ICU.

Blood cultures were negative for bacterial pathogens. Because of anuria, urine cultures were not possible, and albuminuria could not be tested. Examination of peripheral blood smears showed no parasites on admission to ICU and titers of antibodies to leptospira were low.

Microscopy examination of postmortem liver biopsies showed histopathologic changes characteristic of YF: midzonal necrosis with a small rim of a few viable periportal and pericentral hepatocytes and centrilobular cells with microsteatosis and eosinophilic degeneration with round, eosinophilic cytoplasmic structures (Councilman bodies).

A serum sample collected before death and a serum sample obtained from the patient in 1994 during a seroepidemiologic study on HTLV-I infection and stored at -80°C at the Institut Pasteur de la Guyane, French Guiana, were tested for immunoglobulin (Ig) G and IgM specific for three flaviviruses (YF, dengue, and Saint Louis encephalitis), two alphaviruses Tonate and Mayaro), and a new world

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arenavirus (Tacaribe), by enzyme-linked immunosorbent assay (3). A plaque reduction neutralization test was also used to detect YF-neutralizing antibodies (4). The serum collected in 1998 contained IgM (but not IgG) that specifically recognized YF antigens. IgM specific for other flavivirus antigens (dengue, Saint Louis encephalitis) were not found. The neutralizing antibody titer of the 1998 serum as assessed by plaque reduction neutralization test was 20. In the serum collected in 1994, no YF virus–specific antibodies were detected by any technique. IgG to Mayaro virus was detected in both sera; no other antibodies to alphaviruses and arenaviruses were detected.

Serum and homogenized liver samples from the patient were diluted 10-fold in Leibowitz medium containing 3% fetal calf serum, and dilutions were injected into subconfluent AP 61 cell cultures (5). After 7 days, cells were harvested and tested for YF virus by an indirect immunofluorescence assay using a monoclonal antibody from the Centers for Disease Control and Prevention, Fort Collins, CO, USA. YF virus was not isolated by cell culture from either blood or liver samples.

Reverse transcription–polymerase chain reaction (RT-PCR) tests were performed on RNA extracted from serum and liver (6). RNA of the YF virus was detected by RT-PCR after seminested PCR only in the liver sample. The 542-bp PCR product was purified and directly sequenced with an automatic sequencing system (ACTgene, EuroSequence Gene Services, Evry, France). The first 309 nucleotides of the 3' noncoding sequence were aligned with those of sequenced YF strains from Genbank (7,8). Sequences were aligned with the Clustal W program. Bootstrap confidence limits were calculated from 100 replicates with the program SEQBOOT. Phylogenetic analyses were performed by maximum parsimony by using the DNAPARS program with uniform character weights and a heuristic search option. All branch lengths were drawn to scale by the program Treetool. The sequence of the YF strain isolated in 1998 was deposited in Genbank (accession number: AF121952).

The sequence of the amplified gene differed at 11 positions (3.5% nucleotide divergence) from that of a Brazilian strain isolated in 1935 and at 21 positions (6.8% nucleotide divergence) from that of a strain isolated in Ecuador in 1979. The sequence diverged more from strains isolated in Peru (1995) and Trinidad (1979) (8.1% and 10% divergence, respectively). As expected, African strains differed more, with those of West Africa (from Nigeria and Senegal) being less distant than those from East and Central Africa (Uganda and Central African Republic). The nucleotide sequence downstream from the NS5 stop codon in the 3' noncoding region of the YF virus was deleted in the French Guianese strain, as in several South American YF viruses (9). Phylogenetic analysis of the sequences confirmed that the virus was most closely related to those isolated in Brazil and Ecuador (Figure).

Conclusions

The histopathologic changes of the liver were characteristic of YF but also of other hemorrhagic fever viruses. The IgMs were only slightly above the cut-off values used in the laboratory. Although YF was highly probable, the diagnosis required confirmation by detection of the virus or its genome. Indeed, in 1990 a suspected case of YF was reported to the World Health Organization because of characteristic histopathologic changes, but the case was never confirmed (presence of IgG but no IgM specific for YF, no detection of the virus in liver or serum); later it was shown that the patient had been vaccinated against YF 1 year before (10). However, because YF requires health authorities to take specific measures, confirmation of the diagnosis is important, especially when YF is not prevalent. The case we described was confirmed by RT-PCR from liver only, because the serum sample was taken on day 6 when viremia is usually resolved and because the sensitivity of cell culture for virus in liver samples is very low (probably because of biliary salts toxicity). This case underscores the need for postmortem liver biopsies for detecting the viral genome to confirm diagnosis.

This patient did not leave her village the months before infection; she was probably infected while working in forest clearings. The detection of a YF virus in French Guiana nearly a century after the last report is notable; however, the absence of reported cases during the previous years is surprising because no natural borders exist between this country and northern Brazil, where YF is not uncommon (11). A severe YF outbreak would have easily been detected, but sporadic cases can be misdiagnosed

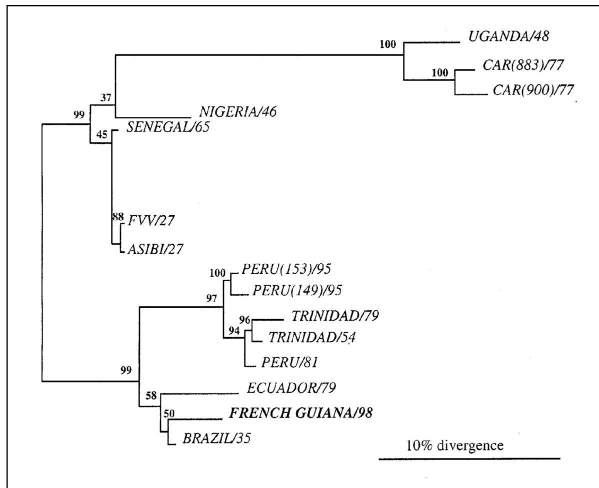

Figure. Phylogenetic tree generated from 309 nucleotides of the 3' noncoding region of the strain of yellow fever (YF) isolated in French Guiana in 1998 (in bold) and of 14 other YF strains by using the DNAPARS program. Numbers indicate bootstrap values for groups to the right. One µl (30 ng) of primer VD8 (5'-GGGTCTCCTCTAACCTCTAG-3') was mixed with RNA resuspended in 10 µl of distilled water; the mixture was heated at 95°C for 2 minutes and placed on ice. cDNA was synthesized in a mixture containing reverse transcriptase (RT) incubation buffer (provided by the manufacturer), 0.2 mM of each of the four dNTPs, 20 units RNasine, and five units of AMV RT (Promega, Charbonnières, France) by incubation for 1 hour at 42°C. cDNA was amplified by PCR. Four µl of the cDNA sample was added to 46 µl of a mixture containing Taq polymerase buffer (provided by the manufacturer), 2 mM $MgCl_2$, 0.5 mM of each of the 4 dNTPs, 300 ng of primer VD8 and of degenerate primer EMF1 (5'-TGGATGACSACKGARGAYATG-3') (S = C, G; K = G, T; R = A, G; Y = C, T), and 0.5 unit of Taq polymerase (Promega, Charbonnières, France). After 5 minutes of denaturation at 95°C, the mixture was subjected to 30 polymerase chain reaction (PCR) cycles: 95°C for 30 seconds, 53°C for 90 seconds, and 72°C for 60 seconds, followed by a final 10-minute polymerization step at 72°C. Four µl of a 1 in 100 dilution of the PCR products was used for seminested PCR using primers VD8 and NS5YF (5'-ATGCAGGACAAGACAATGGT-3'). After the denaturation step, DNA was amplified by 25 cycles of PCR: 94°C for 30 seconds, 55°C for 90 seconds, and 72°C for 120 seconds, followed by a final extension step at 72°C. Negative controls (serum from healthy persons) were included in the series. The positive control (supernatant of infected mosquito cells) was tested separately to avoid any contamination. The phylogenetic analysis was conducted by the Pasteur Institute in Paris.

as other fevers or as hepatitis (when jaundice is present) and may be not tested for YF even though serologic and YF virus detection tests are performed for each suspected case.

No other case was diagnosed in the patient's family or neighborhood, but sporadic cases are common in South America (12), probably because of poorly anthropophilic vectors. Furthermore, in this area, approximately 90% of the population have been vaccinated at least once (R. Pignoux, unpub. data). Outbreaks are common among nonhuman primates, but no epidemic has occurred in the area where the patient lived. However, YF incidence increased in northern Brazil in 1998 (13).

This case calls attention to vaccination problems in French Guiana, especially along the rivers. Our patient had been vaccinated in 1985, but the absence of neutralizing antibodies in 1994 indicates that the vaccination was not effective. Although this patient may have had a poor antibody response, more likely inadequate storage of the vaccine before use was responsible. In 1985, YF vaccines were less thermostable than now, and the cold chain was difficult to maintain. In response to this case, an immunization campaign was initiated in this area in May.

Vaccination of the population must continue since YF can reappear. The immunization program implemented in French Guiana (compulsory vaccination of children older than 1 year of age, booster YF vaccination every 10 years, and required vaccination certificate before entering school) should avert the threat of outbreaks in urban areas, which have a vaccine coverage rate of approximately 80%. However, the risk for sporadic cases in unvaccinated persons will persist, and so active serologic and virologic surveillance of YF remains necessary.

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Risk for Rabies Transmission from Encounters with Bats, Colorado, 1977–1996

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To assess the risk for rabies transmission to humans by bats, we analyzed the prevalence of rabies in bats that encountered humans from 1977 to 1996 and characterized the bat-human encounters. Rabies was diagnosed in 685 (15%) of 4,470 bats tested. The prevalence of rabies in bats that bit humans was 2.1 times higher than in bats that did not bite humans. At least a third of the encounters were preventable.

Although no cases of human rabies have been reported since 1931 in Colorado, rabies remains a health risk in this state because of the frequency with which Coloradans have contact with bats. The first objective of this study was to determine the prevalence of rabies in bats that were submitted for laboratory testing in Colorado over a 20-year period, including an analysis by bat species. The second objective was to characterize the circumstances of confirmed bat-human encounters during this same period and to evaluate how this information could be used to prevent human rabies.

Data Sources

Laboratory Records

Rabies diagnosis was conducted by two laboratories in Colorado: the Colorado Department of Public Health and Environment (CDPHE) Laboratory and the Colorado State University (CSU) Veterinary Diagnostic Laboratory. Bats were accepted for testing from public and private sources if they had had contact with a person or a domestic pet, if the possibility of contact could not be excluded, or if the bat exhibited abnormal behavior. County agencies were also permitted to submit up to three bats per week (usually found dead from no apparent cause or exhibiting aberrant behavior) for local surveillance. None of the submissions were for studies of rabies prevalence among bats with a normal appearance or in their natural habitat.

Records from both laboratories were maintained by the CDPHE Epidemiology Division and made up the first dataset we analyzed. Information extracted from these records included rabies test date, test result, and bat bite information. For bats sent to CDPHE (but not CSU), laboratory technicians identified the bats by species, and the data were included in the analysis.

Possible Rabies Exposure Memoranda

A second dataset consisted of memoranda describing any animal exposure reported to CDPHE resulting in rabies postexposure prophylaxis (PEP). Memoranda were not written for encounters in which the animal tested negative for rabies, even if a person was bitten. Infrequently, CDPHE staff wrote memoranda before learning that an animal had tested negative for rabies or when PEP was recommended but not administered. Four persons in the Epidemiology Division worked on zoonosis control during the 20-year study period; two of them wrote 90% of the memoranda. A bat encounter was defined as bat contact or possibility of bat contact with a person. A wound was defined as a visible puncture, scratch, bleeding, or a sensation of sharp pain during the encounter. No attempt was made to distinguish bite wounds from claw marks or scratches.

The analysis of circumstances was restricted to memoranda in which the presence of a bat was

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documented. Memoranda that described encounters with bats were identified, and data on person, place, and time were extracted. The circumstance of encounter was listed as one of 13 general categories that best described the event. Any person who initiated the standard rabies PEP series was designated as having received treatment.

Findings

Laboratory Records

From 1977 through 1996, 4,502 bats were submitted for testing. CDPHE received 4,394 bats (98%), and CSU received 108 bats (2%). These bats represented 15 (83%) of 18 species present in Colorado (1) (Table 1). Thirty-two bats were excluded from further analysis because either the test result or the bite status was unrecorded. Rabies was diagnosed in 685 (15%) bats and accounted for 98% of all animal rabies cases in Colorado during the study period. Of the 233 bats that bit people, 69 (30%) had rabies. Of the 4,237 bats that did not bite people, 613 (14%) had rabies. The prevalence of rabies among bats that bit humans was 2.1 times higher (95% confidence interval 1.7 to 2.5) than in bats not involved in human bites. None of the persons bitten by bats got rabies. Human rabies has not been reported in Colorado since 1931 (CDPHE, unpub. data, 1998).

Species data were available for 4,182 (94%) bats. Three species—big brown bats (*Eptesicus fuscus*), hoary bats (*Lasiurus cinereus*), and silver-haired bats (*Lasionycteris noctivagans*)—accounted for 73% of total submissions, 84% of the rabies-positive specimens, and 70% of bats involved in bite incidents. The prevalence of rabies in silver-haired bats (5%) was lower than in big brown bats (17%) or hoary bats (40%).

Table 1. Prevalence of rabies in bats submitted for testing, Colorado, 1977–1996

Species	No. that bit humans (% rabid)	No. that did not bite humans (% rabid)	Total no. tested (% rabid)
Big brown bat *Eptesicus fuscus*	122 (27)	2,013 (16)	2,135 (17)
Myotis genus group[a]	35 (14)	722 (6)	757 (7)
Silver-haired bat *Lasionycteris noctivagans*	28 (14)	628 (5)	656 (5)
Hoary bat *Lasiurus cinereus*	13 (77)	452 (39)	465 (40)
Long-eared bat *Myotis evotis*	8 (88)	38 (21)	46 (33)
Brazilian free-tailed bat *Tadarida brasiliensis*	0 (0)	41 (12)	41 (12)
Red bat *Lasiurus borealis*	1 (100)	25 (8)	26 (12)
Pallid bat *Antrozous pallidus*	0 (0)	21 (5)	21 (5)
Big free-tailed bat *Nyctinomops macrotis*	2 (50)	19 (11)	21 (14)
Townsend's big-eared bat *Plecotus townsendii*	1 (0)	13 (0)	14 (0)
Species data unavailable	23 (35)	265 (9)	288 (11)
Total	233 (30)	4,237 (14)	4,470 (15)

[a]Includes six species in the genus *Myotis* that could not be easily distinguished by inspection: *M. lucifugus, M. volans, M. thysanodes, M. californicus, M. ciliolabrum,* and *M. yumanesis.*

Memoranda

During the 20-year study period, 271 memoranda described possible encounters with bats; 240 (89%) memoranda documented the presence of a bat and were included in the analysis. Of the 131 bats tested, 99 had rabies.

Of the 240 persons who encountered bats, 141 (59%) were male and 99 (41%) were female. From the 195 (81%) records that recorded the person's age, the range in age was 10 months to 81 years, and the median age was 25 years. Of the 182 (76%) persons reporting that they were wounded, the most common wound site was the hand (59%), followed by the arm (14%), head/neck (12%), leg/foot (9%), torso (2%), or multiple sites (2%).

Not enough information was available to characterize the time of day of the encounters. Two hundred (83%) encounters occurred between June and September, corresponding to peak activity periods and seasonal migratory patterns of bats in Colorado.

In the 217 records that noted location of bat encounters, 117 (54%) occurred outdoors, chiefly on home properties and park and recreation areas (none were reported in caves). Of the 100 (46%) bat encounters inside buildings, 83 were in private homes (37 of these in bedrooms). Big brown bats, colonial bats that commonly roost

inhouses and buildings, were encountered more frequently outside than inside (60% vs. 39%) when rabid (n = 46). All 11 rabid hoary bats, solitary tree dwellers, were encountered outdoors. However, rabid silver-haired bats, another solitary tree-roosting species, were encountered equally indoors (n = 3) and outdoors (n = 3).

The four most frequent circumstances in which people encountered bats, accounting for 62% of the encounters, were a bat landing on an awake person (19%), a person picking up a grounded bat outside (18%), a person awakening to find a bat in the room (15%), and a person trying to remove a bat from inside a structure (10%). The remaining nine circumstances occurred repeatedly but less frequently (Table 2).

Of the 240 persons who had encounters with bats, 216 (90%) initiated PEP; nine of these stopped treatment after the bat tested negative for rabies. The bat tested negative in 17 of the 24 cases in which PEP was not administered, but a memorandum was written before the test results were available. The remaining seven persons did not receive prophylaxis because they or their physician did not believe that the contact warranted treatment. In three of these seven encounters (which occurred before 1983), the bat was found to be rabid, but no definite wound was observed.

The time from bat encounter to initiation of treatment could be calculated for 199 (92%) of the 216 patients who received PEP and was 1 hour to 28 days. Fifty percent of patients received their first dose of vaccine within 24 hours of exposure; 75% started treatment within 72 hours. Of the 18 patients who initiated treatment 7 or more days after the encounter, nine did not do so until advised by an acquaintance or physician of the possible rabies risk.

Table 2. Circumstances in which humans encountered bats, Colorado, 1977–1996

Circumstances	Bat captured and tested: Rabid	Bat captured and tested: Not rabid	Bat not tested	All encoun-ters
Bat landed on person	17	2	27	46
Person picked up bat outdoors	24	5	15	44
Person awoke to find bat in room	17	4	14	35
Person tried to remove bat from indoors	5	2	17	24
Person inadvertently touched hidden bat	3	5	8	16
Person handled captured bat	12	0	1	13
Child found alone with bat	4	3	2	9
Person handled bat as part of job	6	1	1	8
Person stepped on bat	3	0	3	6
Person bitten while taking bat from pet	2	1	3	6
Person bitten by pet that had bat in mouth	1	4	1	6
Person attributed wound to bat they saw	0	2	0	2
Other circumstances	0	0	6	6
Unspecified in report	5	3	11	19
Total	99	32	109	240

Silver-Haired Bats

Although the silver-haired bat rabies virus variant was isolated from 15 of the 21 persons who died of bat-associated rabies in the United States from 1980 through 1997, we observed that silver-haired bats in Colorado had neither the greatest frequency nor the highest species-specific rate of rabies. Our findings are consistent with tabulations from New York (1988 to 1992) and Arkansas, Virginia, and West Virginia (1990 to 1994), which showed that silver-haired bats made up a small proportion of bats submitted for rabies testing; only a small number of submitted silver-haired bats were rabies positive (2,3). Nonetheless, Arkansas (1991), New York (1993), and West Virginia (1994) each had human cases associated with the rabies virus variant common to silver-haired bats (4-6). Because the frequency of human encounters with this species is apparently low and the prevalence of rabies in tested silver-haired bats is small, other factors must explain the silver-haired bats' association with human rabies cases. One hypothesis is that silver-haired bats are more aggressive than other bats (1). Additionally, one study has demonstrated that the rabies virus variant of silver-haired bats replicates in nonneuronal tissue more efficiently than a coyote rabies virus variant (7). This attribute might explain how a small dermal inoculum of silver-haired variant rabies virus from a seemingly superficial bite could cause infection. As silver-haired and hoary bats are tree dwellers that favor old growth forest habitat, it should be unexpected to encounter them indoors. None of the 12 hoary bats (11

rabid) included in this series were encountered inside. In contrast, three of the nine encounters with silver-haired bats were indoors. All three bats were rabid.

Conclusions

Bats that interact with humans are far more likely to have rabies than bats that avoid humans, and rabies prevalence is highest in bats that bite. Conversely, rabid bats appear to interact more frequently and to be more prone to bite than nonrabid bats. This behavior is consistent with clinical manifestations of rabies in wildlife species in which the animal exhibits abnormal behavior, loses its natural fear of humans, and acts aggressively (8).

Encounters with bats resulted from a relatively small number of recurring situations. At least a third of the encounters (picking up grounded bats, handling captured bats, and trying to remove bats from structures or from pets' mouths) were preventable; however, most encounters in which a person inadvertently touched a hidden bat or a bat landed on a person were probably unavoidable.

Delays in treatment suggest that some people may not be aware of the risk for rabies transmission from contact with a bat. In the United States, in nearly half of the cases of human rabies associated with bat variant rabies virus, the person had no history of contact with a bat (9-11). Although unrecognized exposures may have occurred, the persons involved probably did not understand the risk after exposure to a bat and therefore did not seek medical care. Even when specifically asked about animal exposures, some patients and their families initially did not report bat contact (11,12).

This study has several potential limitations. First, we do not know whether the prevalence of rabies in tested bats is representative of all bats that encounter humans. The laboratory testing was a passive surveillance system, dependent on the submission of bats by persons involved in encounters. We calculated a lower limit estimate of the prevalence of rabies among bats that bit humans by using data from the memoranda. If one assumed that the bats that bit humans and later escaped and thus were not tested (90) were rabies-free, the prevalence of rabies among bats that bit people would decrease from 30% (69 of 233) to 21% (69 of 323), still significantly higher than the prevalence in bats that did not bite people ($p < .001$).

The circumstances were categorized for a small proportion of all bat encounters. Memoranda were written for only 131 of 4,502 bats submitted for testing and for 109 encounters in which the bat escaped. The number of unreported encounters cannot be estimated, and information from those encounters could alter the frequencies of the type of encounter presented in this study.

Finally, administration of rabies PEP is not reportable in Colorado and, therefore, the study may not have included all persons who received PEP after a bat encounter. Because the state health department was the primary source of rabies biological supplies for medical providers in the state from 1977 through 1985, nearly all exposures requiring PEP would have come to the department's attention. Rabies biological supplies were more widely available from other sources after 1985. Although the number of reported PEP administrations remained stable, some exposures may not have been reported.

Two findings of this study support recent revisions of the Advisory Committee on Immunization Practices (ACIP) recommendations (13-15): bats that encountered humans had a high prevalence of rabies, and the third most frequently reported circumstance was a person awakening to find a bat in the room. The ACIP stated in October 1997 that PEP may be appropriate even in the absence of demonstrable bite, scratch, or mucous membrane exposures in situations in which such exposure is likely to have occurred (e.g., a sleeping person awakes to find a bat in the room or an adult finds a bat in a room with an unattended child, a mentally deficient person, or an intoxicated person) (14). Of 35 instances reported in this study in which a bat was found in the room by a person upon awakening, 17 bats were rabid, and 23 persons had evidence of a bite.

The Colorado Department of Public Health and Environment, the Colorado Division of Wildlife, and the Colorado Bat Society recently collaborated to publish an educational pamphlet that describes methods to prevent rabies exposure from a bat and measures to take if a person encounters a bat (16).

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Australian Bat Lyssavirus Infection in a Captive Juvenile Black Flying Fox

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The newly emerging Australian bat lyssavirus causes rabieslike disease in bats and humans. A captive juvenile black flying fox exhibited progressive neurologic signs, including sudden aggression, vocalization, dysphagia, and paresis over 9 days and then died. At necropsy, lyssavirus infection was diagnosed by fluorescent antibody test, immunoperoxidase staining, polymerase chain reaction, and virus isolation. Eight human contacts received postexposure vaccination.

Australia was considered free of rabies and the rabieslike viruses of the genus *Lyssavirus* until the recognition in 1996 of Australian bat lyssavirus (ABL) as the cause of a rabieslike disease in a black flying fox *(Pteropus alecto)*(1) and a wildlife caretaker (2). While serotypic, antigenic, and sequence analysis show that ABL is closely related to classic rabies virus and European bat lyssavirus (1), phylogenetic analysis has clearly demonstrated that ABL represents a new genotype, genotype 7 (3). Australia is still considered free of terrestrial (genotype 1) classic rabies (4). Rabies vaccine and antirabies immunoglobulin protect laboratory animals against ABL infection (5), and their use pre- and post-ABL exposure is recommended for humans (6,7).

On the morning of December 8, 1997, two juvenile black flying foxes were found clinging to each other and vocalizing in a residential area near an urban flying fox colony. An experienced, rabies-vaccinated wildlife caretaker retrieved the two animals from an unusually low tree roost. On the basis of body weight and forearm measurements, their age was estimated at 2 to 3 weeks, an age of total maternal dependency. Their physical condition was normal.

Both animals, Bat 1 (male) and Bat 2 (female), remained with the original caretaker for 2 days before being placed with two different caretakers for hand-rearing. Bat 1 was communally housed with another orphaned black flying fox, and Bat 2 was housed with two others. For the next 5 weeks, all the bats were clinically normal. However, in week 6, Bat 1 began to exhibit signs of neurologic disease. The caretaker first observed the bat's sudden and progressive aggression toward its companion and separated them. Throughout day 1 of illness, the bat periodically "frothed at the mouth" and had repeated lordotic spasms, during which it vocalized loudly. Treatment with oral amoxycillin was initiated. On day 2, the bat was calmer but still vocal, attempting to bite objects and eating little. On day 3, it was no longer aggressive and was only able to eat pulped food and milk. On day 4, it was seen by a veterinarian, who noted severe pharyngitis, and administered injectable dexamethasone. The bat was much more alert that evening and ate solid food well. The dexamethasone injection was repeated on day 5; the bat remained alert and ate solid food overnight. On day 6, it was dysphagic and was again offered pulped foods and liquids. On days 7 and 8, it was unable to roost normally, lay supine, was progressively dysphagic, had diarrhea, and was losing weight. On day 9, it rapidly got worse and died. The carcass was submitted to the Queensland Department of Primary Industries Animal Research Institute for necropsy.

The emaciated carcass had poor pectoral muscle development and no perirenal, pericardial, or mesenteric fat reserves. ABL infection was diagnosed by fluorescein-labeled antirabies

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monoclonal globulin (CENTOCOR) in a fluorescence antibody test (FAT) on impression smears of fresh brain. With the absence of other lyssaviruses in Australia (1), a positive reaction with this lyssavirus genus-specific antibody is considered diagnostic for ABL. Sections of brain showed nonspecific, nonsuppurative meningoencephalitis with perivascular cuffs of mononuclear cells and widespread focal gliosis. Numerous neurons contained eosinophilic inclusion bodies, which are highly suggestive of lyssavirus infection.

Immunoperoxidase staining of formalin-fixed, paraffin-embedded sections of brain with a monoclonal antinucleoprotein antibody (Clone HAM, provided by R. Zanoni, Berne University, Switzerland) detected lyssavirus antigen in neurons of the frontal cortex, hippocampus, brain stem, and cerebellum, including Purkinje cells. This antigen distribution is consistent with previous reports of rabies (8). The diagnosis was independently confirmed at the Commonwealth Scientific and Industrial Research Organisation's Australian Animal Health Laboratory by FAT, immunoperoxidase staining, virus isolation in murine neuroblastoma cells, and sequence analysis of polymerase chain reaction (PCR) product (P. Daniels, pers. comm.). No blood was available for serologic testing.

After ABL infection was diagnosed in Bat 1, the Brisbane Southside Public Health Unit received information that up to eight persons had been bitten or scratched by the bat in the weeks before and during its illness. Six were bat handlers who had received postexposure treatment with five doses of rabies human diploid cell vaccine (HDCV) during a 1996 campaign that followed the diagnosis of the first human case of ABL (2,7). Two were unvaccinated members of the principal bat caretaker's household. Despite recommendations that unvaccinated members of bat caretakers' households not handle bats, the two had come into regular contact with the bat and may have been scratched during that time.

Lyssavirus prophylaxis was commenced in accordance with Australian recommendations (6,7). All eight persons provided blood for rabies serologic testing (indirect-enzyme linked immunosorbent assay [ELISA]). The six vaccinated bat handlers had titers of 1.130 IU/ml to >8.80 IU/ml 12 months after their initial vaccinations (World Health Organization–recommended protective level for rabies = 0.5 IU/ml [9]). Each of these received two further intramuscular doses of 1.0 ml of HDCV, according to the Australian recommendations for postexposure treatment of vaccinated persons. The two unvaccinated persons had titers of <0.13 IU/ml (nonimmune) and received the standard postexposure treatment for unvaccinated persons: 20 IU per kg of human rabies immune globulin (HRIG) by intramuscular injection and five intramuscular doses of 1.0 ml of HDCV. All eight remain well 10 months after the incident.

Throughout this episode, Bat 2 and the other three bats that had been directly or indirectly in contact with the infected bat remained healthy. After the diagnosis of ABL in Bat 1, these four animals were quarantined for observation at the Animal Research Institute for 11 weeks and then euthanized. All were antibody-negative (<0.5 IU/ml) by rabies rapid fluorescent focus inhibition test when quarantined and remained so during the observation period. Brain impression smears from the four were negative for lyssavirus antigen by FAT.

This case of naturally occurring ABL infection is of particular interest for several reasons. First, the astute observations of the bat caretaker provide possibly the first record of the clinical course of natural ABL infection in a flying fox. Second, to our knowledge, this is the first recorded case of ABL disease in a maternally dependent juvenile. Third, the case history provides the first indication of the incubation period and length of clinical disease in naturally infected flying foxes. Natural in utero infection with lyssaviruses is not known to occur (10), and the four flying foxes in contact with Bat 1 were unlikely to be the source of infection as they subsequently tested negative for ABL antibody and antigen. The infection appears to have occurred in the 2 to 3 weeks before the rescue and, after an incubation period of 6 to 9 weeks, produced 9 days of clinical disease. Infection in Bat 1 most probably resulted from a bite from an ABL-infected bat. This bat may have been Bat 1's dam.

This episode demonstrates the necessity of examining for ABL any flying fox that has bitten or scratched a person and of improving community and professional awareness of the disease and associated risks. Costly postexposure treatment with HRIG can be

avoided if only vaccinated persons handle Australian bats.

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Bordetella holmesii-Like Organisms Isolated from Massachusetts Patients with Pertussis-Like Symptoms

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We isolated *Bordetella holmesii*, generally associated with septicemia in patients with underlying conditions, from nasopharyngeal specimens of otherwise healthy young persons with a cough. The proportion of *B. holmesii*-positive specimens submitted to the Massachusetts State Laboratory Institute increased from 1995 to 1998.

Bordetella holmesii is a recently described gram-negative, asaccharolytic, nonoxidizing, soluble, brown-pigment-producing rod previously known as CDC nonoxidizer group 2 (NO-2) (1). This group consists of 15 closely related, biochemically similar strains of fastidious nonmotile bacteria isolated from human blood cultures. In establishing NO-2 as a species, Weyant et al. (1) performed 16S rRNA sequencing of one NO-2 strain and the type strains of *B. pertussis, B. parapertussis, B. bronchiseptica,* and *B. avium*. They found a high degree of homology among them (≥98% over 1,525 bases) and confirmed a close relatedness between NO-2 and *Bordetella* species by DNA relatedness studies (hydroxyapatite method). Biochemically, the lack of oxidase activity and the production of a brown soluble pigment differentiate *B. holmesii* from *B. pertussis, B. bronchiseptica,* and *B. avium*; the lack of urease activity differentiates it from *B. parapertussis* (1).

Unlike *B. pertussis*, which causes whooping cough, *B. holmesii* has been associated most often with septicemia in patients with underlying conditions (1-4). It also has been isolated from sputum from one patient with respiratory symptoms (3). Van den Akker (5) suggested that the difference in lipopolysaccharide expression (important in bacterial pathogenesis) between the closely related *B. pertussis* and *B. holmesii* might help explain their observed difference in propensity to infect respiratory tract epithelium verses causing opportunistic bacteremia.

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In Massachusetts, however, we have seen *B. holmesii* associated with a different clinical picture. From January 1995 (when the article describing *B. holmesii* [1] was published) through December 1998, the Massachusetts State Laboratory Institute (SLI) isolated *B. holmesii* from 34 clinical specimens: 33 nasopharyngeal specimens from patients suspected of having pertussis and one blood culture specimen from a 45-year-old patient with septicemia. Of the 33 patients with respiratory symptoms, 30 (91%) were 11 to 29 years old, 1 (3%) was an infant, and 2 (6%) were 10 years old; most were otherwise healthy.

B. holmesii is confirmed by its biochemical patterns and cellular fatty acid analysis or the DNA transformation test will definitively separate *B. holmesii* from *Acinetobacter*, neither procedure is performed at SLI. Three of the initial isolates were sent to the Centers for Disease Control and Prevention, Atlanta, Georgia, for definitive identification and were confirmed as *B. holmesii* by cellular fatty acid analysis. The remaining 31 isolates were biochemically and morphologically identical to those.

B. holmesii–positive nasopharyngeal specimens have increased both in absolute number and as a percentage of the total nasopharyngeal specimens processed at SLI (Table). The number rose from 3 (0.1% of total specimens submitted for pertussis culture) in 1995, to 6 (0.2%) in 1996,

Table. *Bordetella* species isolated from nasopharyngeal (NP) specimens at the Massachusetts State Laboratory Institute, 1994–1998

	1994		1995		1996		1997		1998	
	No.	%	No.	%	No.	%	No.	%	No.	%
B. pertussis	75	4.2	140	5.8	325	8.9	132	5.6	165	6.6
B. parapertussis	7	0.4	20	0.8	32	0.9	11	0.5	NA	NA
B. holmesii	0	0	3[a]	0.1	6	0.2	9	0.4	15	0.6
Total NP specimens reported	1,792		2,399		3,653		2,375		2,508	

[a]Does not include the one case of *B. holmesii* isolated from blood.

to 9 (0.4%) in 1997, and to 15 (0.6%) in 1998. A chi-square analysis for linear trend of proportions from 1995 to 1998 found the trend significant (chi-square = 11.6, p <.001), possibly indicating a rise in prevalence. (When such an analysis was applied to the proportion of nasopharyngeal specimens testing positive for pertussis during the same period, no trend was apparent ([chi-square = 0.8, p = .36].) A growing awareness of the species by laboratory personnel may be contributing to the observed increase.

To ascertain what symptoms were associated with *B. holmesii* isolation, we investigated the 23 cases identified in the 24 months from January 1997 through December 1998. We called providers and patients for disease histories and demographic information. Pertussis case report forms, modified to include more possible symptoms and underlying conditions, were used to record the information collected in the interviews.

Nineteen (83%) of the 23 *B. holmesii*-positive cases were in adolescents (11 to 19 years), 2 (9%) in young adults (20 and 29 years), 1 (4%) in a 10-year-old child, and 1 (4%) in an infant. All had cough. In addition, 14 (61%) had paroxysms, 2 (9%) had whoop, and 6 (26%) had posttussive vomiting. No other symptoms were identified by patients or providers. Fourteen (61%) of the 23 had no underlying conditions, 8 (35%) had minor conditions such as occasional asthma or allergies, and 1 (4%) had chronic fatigue.

The fact that cultures were taken from 20 (87%) of the 23 case-patients within 14 days of cough onset generally excluded convalescent-stage pertussis as a cause of symptoms. However, *B. pertussis* had been confirmed in a 14-year-old girl, who had occasional asthma, 3 months before *B. holmesii* was confirmed—she received a reculture because of a persistent cough that had not resolved since the original infection. She had had paroxysms and vomiting associated with the pertussis infection but no symptoms other than cough at the time *B. holmesii* was isolated. Cultures were taken on the same day from two sisters, 15 and 9 years old, each with cough of fewer than 14 days. *B. holmesii* was culture-confirmed in the 15-year-old; *B. pertussis* was culture-confirmed in the 9-year-old. This raises the question of whether *B. pertussis* and *B. holmesii* might cocirculate.

At least 11 (48%) of the 23 cases were found during active surveillance for pertussis in school and university settings, which may explain the age profile of the cases. Three case-patients, with cough onset dates of April 1, 1997; February 27, 1998; and March 9, 1998, were students at the same university. These cases, though not epidemiologically linked to each other, were in symptomatic contacts of pertussis patients and were cultured as part of an azithromycin efficacy study. At least eight other cases were also in contacts of confirmed patients with pertussis and were detected through active surveillance. No cases were definitively linked. Peak months of cough onset were November and December (8 of the 23 cases), as is true for pertussis in Massachusetts, with a smaller peak in March and April (6 of the 23 cases). The observed peak in November-December may be due to the role of active surveillance for pertussis in ascertaining cases of *B. holmesii* colonization.

The clinical profile of the 21 cases in adolescent and adult patients infected with *B. holmesii* was compared with that of 122 culture-confirmed pertussis cases in patients between 11 and 29 years of age with cough onsets in the same period, i.e., 1997 through 1998. (Relevant clinical information was not available for an additional 42 culture-confirmed pertussis case-patients in this age group.) We did not consider cough duration, because of imprecise data, but rather focused on the presence or absence of three classic pertussis symptoms: paroxysms of cough, whoop, and posttussive

vomiting. Cases were categorized as patients with 0, 1, or 2 to 3 of these symptoms. (No separate category for three symptoms was used due to a cell size of 0 in the case of *B. holmesii*.) On applying the chi-square test for independent proportions, we found *B. holmesii* infection milder (i.e., accompanied by fewer of the above three pertussis symptoms) than *B. pertussis* infection (chi-square = 10, $p < .01$).

To rule out the possibility that the difference was due to more frequent cultures for severe than for milder pertussis cases, we compared the 21 adolescent and adult *B. holmesii* patients with the 577 SLI–serology-positive pertussis patients 11 to 29 years of age with cough onsets in 1997 or 1998 for whom sufficient clinical data were available. The SLI pertussis serology test is a single-serum enzyme-linked immunosorbent assay for immunoglobulin G to pertussis toxin, available since 1987. The assay is for use in persons ≥ 11 years of age and is optimally sensitive at 2 to 8 weeks after cough onset. By the same methods as for the previous comparison, we found that the *B. holmesii* cases were milder at a higher level of significance (chi-square = 69, $p < 10^{-8}$).

Without knowing the prevalence of *B. holmesii* carriage in asymptomatic persons, we cannot say with certainty that *B. holmesii* is the causative agent for the respiratory symptoms of the patients from whom it was isolated. Approximately half the cases were discovered through active surveillance for pertussis. This, together with the fact that the symptoms associated with *B. holmesii* were relatively mild, suggests that the organism may not have been causing disease. On the other hand, *B. holmesii* may be the etiologic agent, given that it is closely related to *B. pertussis* and the associated symptoms (like those of *B. parapertussis)* are similar. *B. pertussis* is the only *Bordetella* species known to produce pertussis toxin, although *B. parapertussis* and *B. bronchiseptica* have silent copies of the toxin gene (6). We do not know whether *B. holmesii* has the toxin gene. However, since *B. parapertussis* can cause disease (albeit not as severe as *B. pertussis* [7]), the presence of pertussis toxin is not necessary for the development of symptoms.

Continued investigation, including conducting diagnostic tests for agents such as *Chlamydia* and *Mycoplasma* and culturing symptomatic and asymptomatic contacts, is warranted to ascertain the degree to which *B. holmesii* is pathogenic in the respiratory system and contagious. If it is contagious, antibiotic susceptibility testing is also needed. *B. holmesii* is susceptible to some 15 antibiotics of a variety of classes (2,3), but whether erythromycin, the drug of choice for pertussis, is effective is not known. SLI is establishing routine erythromycin susceptibility testing of *B. pertussis* and will also test *B. holmesii* isolates.

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New *Cryptosporidium* Genotypes in HIV-Infected Persons

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Using DNA sequencing and phylogenetic analysis, we identified four distinct *Cryptosporidium* genotypes in HIV-infected patients: genotype 1 (human), genotype 2 (bovine) *Cryptosporidium parvum*, a genotype identical to *C. felis*, and one identical to a *Cryptosporidium* sp. isolate from a dog. This is the first identification of human infection with the latter two genotypes.

Protozoan apicomplexan parasites from the genus *Cryptosporidium* infect a wide variety of hosts (1). The parasites are transmitted to humans through contaminated drinking water (2), contact with infected animals, and contact with infected persons (3). In the immunocompetent, cryptosporidiosis manifests itself as self-limited diarrhea, sometimes accompanied by nausea, abdominal cramps, fever, and vomiting. In the immunodeficient, however, cryptosporidiosis may be severe, chronic, and life-threatening (4).

Cross-infection experiments, in which *Cryptosporidium* oocysts were obtained from animals of one species and fed to animals of another species, have investigated the host specificity of this parasite (5). The differences observed in the host range of putative *C. parvum* isolates led to a proposal to establish the *Cryptosporidium* isolate originating from guinea pigs, morphologically indistinguishable from *C. parvum*, as a new species—*C. wrairi*—solely on the basis of experimental infection (6). The possibility of many *Cryptosporidium* species fostered the development of techniques suitable for typing isolates. Commonly used techniques are isoenzyme analysis (7), Western blotting (8,9), random amplified polymorphic DNA analysis (10-12), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis (13,14), and PCR followed by DNA sequencing (11,15-19).

Early studies of the polymorphism of isolates classified as *C. parvum* found significant geographic variation among isolates (20) in the region coding for the small subunit ribosomal RNA (SSU-rRNA), commonly used for taxonomic classification. Recently, it has been shown (21) that one of the sequences used in this analysis (22) was erroneously identified as a *C. parvum* sequence, while in fact it was *C. muris*. More recent work (e.g., Le Blancq et al. [23] and GenBank entry AF040725) has shown that the SSU-rRNA region of the *C. parvum* zoonotic (bovine) genotype does not show heterogeneity and is practically identical to the sequence submitted to GenBank in 1993 (accession number L16996, [24,25]).

Recently, consistent results of typing bovine and human *C. parvum* isolates led to unequivocal recognition of two genotypes of *C. parvum*. These two genotypes were reproducibly differentiated by sequencing the SSU-rRNA coding region (16), sequencing the *Cryptosporidium* thrombospondin-related adhesion protein (TRAP-C2) gene (17), and PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene (15). Thus far, the anthroponotic genotype (genotype 1) has been found only in infected humans, while the zoonotic genotype (genotype 2) has been found both in infected humans and in livestock, e.g., cows, lambs, goats, and horses. The published partial SSU-rRNA sequence of

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the *C. parvum* genotype 1 (16) is identical to the *Cryptosporidium* SSU-rRNA sequence that we observed in PCR-amplified DNA from a patient with AIDS (GenBank accession number L16997). That the data from different laboratories for these two genotypes were reproducible calls into question the recent conclusion of Tzipori and Griffiths (26) that "there are no clearly defined and fully characterized reference 'strains' of *Cryptosporidium*."

Other new *Cryptosporidium* genotypes have been identified in various animals (pigs, mice, cats, and koalas) by sequencing the region coding for SSU-rRNA (16,27,28). The cat genotype is thought to represent *C. felis* (28). When morphologic and other data are available for these new genotypes, they may be recognized as new *Cryptosporidium* species.

We present results of typing *Cryptosporidium* isolates by direct sequencing the variable region of the SSU-rRNA amplified from fecal specimens with *Cryptosporidium* genus-specific diagnostic PCR primers (25). Applying this method to a set of specimens from a 3-year longitudinal study on the risk for enteric parasitosis and chronic diarrhea in immunodeficient patients with a low CD4+ count, we found the first human cases of infection by *C. felis* and by a newly identified zoonotic *Cryptosporidium* species possibly originating from a dog.

The Study

All analyzed specimens were collected from January 1991 through September 1994 in a study assessing the impact of enteric parasite-associated diarrhea in persons infected with HIV (29). Study participants answered comprehensive questionnaires concerning clinical and epidemiologic information and provided stool specimens monthly. All stool specimens were examined for *C. parvum* by Kinyoun carbol-fuchsin modified acid-fast stain (30) and direct immunofluorescence (31). Stained slides were examined by observing 200 oil-immersion fields. *C. parvum* was associated with 18 (5.1%) of the 354 acute episodes and 36 (12.9%) of the chronic episodes of diarrhea reported by the participants. All specimens from this study were preserved in separate vials of 10% formalin and polyvinyl alcohol (Para-Pak Stool System; Meridian Diagnostics, Inc., Cincinnati, OH) and thus were not suitable for molecular analysis. Some specimens were originally aliquoted and stored without preservation at -80°C. Of this set, we selected 18 available specimens for 10 randomly chosen *Cryptosporidium*-positive patients for this study.

An aliquot of approximately 300 µl of each stool specimen was suspended in 1 ml of 0.01 M phosphate-buffered saline, pH 7.2, containing 0.01 M of EDTA (PBS-EDTA), and the suspension was centrifuged at 14,000 x g, 4°C for 5 minutes. The pellet from this centrifugation was washed two more times under the same conditions. The pellet was resuspended in 300 µl of PBS-EDTA and used for DNA extraction, performed with the FastPrep disrupter and the FastDNA kit (BIO 101, Inc., Vista, CA) (32). Extracted DNA was stored at 4°C until PCR amplification.

Cryptosporidium genus-specific primers (CPBDIAGF and CPBDIAGR) were used to amplify the *Cryptosporidium* SSU-rRNA variable region (24,25). The conditions for PCR were 95°C for 15 minutes; 45 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 1 minute, 30 seconds; followed by extension at 72°C for 9 minutes; and finished with a hold step at 4°C.

PCR products were analyzed by electrophoresis on 2% SeaKem GTG agarose (Cat. No. 50074, FMC Bioproducts, Rockland, ME), stained with ethidium bromide, and visualized on UV transilluminator. The size of the diagnostic fragment amplified with these primers for *C. parvum* genotype 1 (GenBank accession number L16997) was 438 bp, for *C. parvum* genotype 2 (L16996) 435 bp, for *C. baileyi* (L19068) 428 bp, and for *C. muris* (L19069) 431 bp.

PCR products were purified by using the Wizard PCR Preps kit (Cat. No. A7170, Promega, Madison, WI). Sequencing reactions were done with the Perkin Elmer Big Dye kit (Cat. No. 4303149, PE Biosystems, Foster City, CA) and analyzed on the Perkin Elmer ABI 377 automatic DNA sequencer. Sequences were assembled by using the program SeqMan II (DNASTAR Inc., Madison, WI). Sequences were aligned with the program MACAW (33) and analyzed by programs from the PHYLIP (phylogeny inference program) package (34).

Findings

After the 18 fecal specimens were retrieved from storage and processed for PCR with the

CPBDIAGF/CPBDIAGR diagnostic primer set, visualization of PCR products on 2% agarose gels found two different sizes of diagnostic bands. The size of the *Cryptosporidium* diagnostic bands for patients 53, 75, 119, 124, 153, 278, and 554 did not differ significantly from the size of the standard band (approximately 435 bp) obtained with cloned SSU-rRNA for the *C. parvum* genotype 2 (Figure 1, lane 1). On the other hand, the *Cryptosporidium* diagnostic bands in samples from patients 84, 184, and 485 were visibly larger, approximately 450 bp (Figure 1, lane 3 shows data for patient 84).

DNA sequence analysis showed four types of sequences (Table 1). The sequence of the diagnostic band from patient 53 was 435 bp long and was identical to the corresponding fragment of the *C. parvum* genotype 2 (GenBank accession number L16996) SSU-rRNA. The diagnostic bands in patients 75, 124, 153, 278, and 554 were 438 bp long and were identical to the corresponding fragment of the *C. parvum* genotype 1 SSU-rRNA (GenBank accession number L16997). The sequence from patient 119 was 429 bp long; the diagnostic bands from patients 84, 184, and 485 were 455 bp long and were identical to each other. Sequence similarity searches of GenBank database and molecular phylogeny analysis of the two latter types of sequences showed that, while they were not identical to any sequences in GenBank, they clustered within other representative SSU-rRNA sequences from the genus *Cryptosporidium* (results not shown). In addition, these sequences were significantly different from recently reported partial SSU-rRNA sequences (16,19,27,28). The 429-bp-long diagnostic fragment in patient 119 was identical to a recently identified canine *C. parvum* isolate SSU-rRNA sequence (GenBank accession number AF112576). The longest sequence (455 bp) from patients 84, 184, and 495 was identical in a 100-bp overlap to an updated sequence (GenBank accession number AF097430) for *C. felis* (also called feline *C. parvum* genotype) (28). Alignment of these sequences is shown in Figure 2. In the hypervariable alignment region (from position 101 to 110), all genotypes display a variable number of thymidines. Genotype 1 has the longest stretch, 11 thymidines, while the *Cryptosporidium* sp. (canine genotype) has only one. The sequence for *C. felis* has five insertions at alignment positions 45, 86, 111, 187, and 218, as well as one deletion of two thymidines at positions 197 and 198. Apart from the difference in the hypervariable region, the *Cryptosporidium* sp. (canine genotype) has a deletion of two bases at positions 49 and 50.

Table 1. *Cryptosporidium* genotypes for 10 selected patients in this study

Patient no.	Band size[a] (bp)	*C. parvum* genotype
53	435	Zoonotic (genotype 2)
75, 124, 153 278, 554	438	Anthroponotic (genotype 1)
119	429	*Cryptosporidium* sp. (canine genotype)
84, 184, 485	455	*C. felis*

[a]Size of the *Cryptosporidium* diagnostic band obtained with the CPBDIAGF/CPBDIAGR PCR primer pair

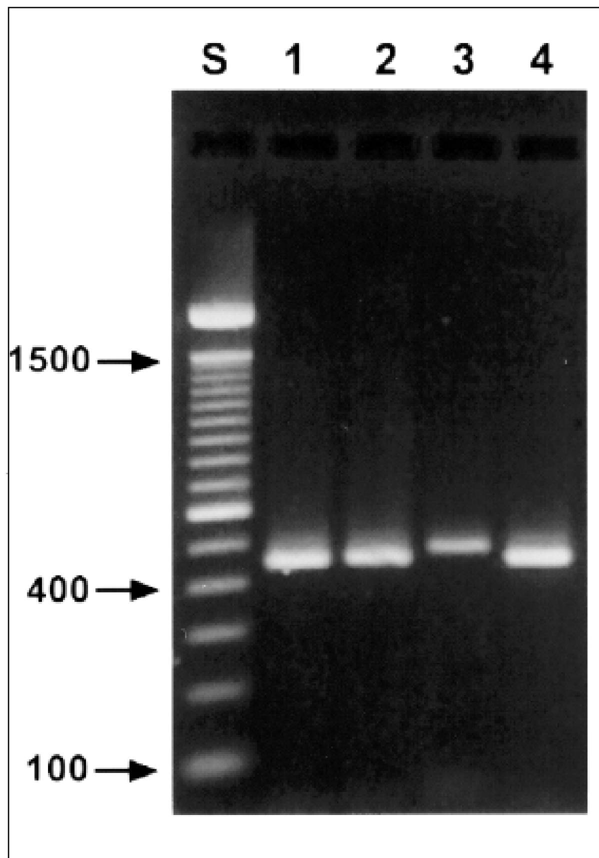

Figure 1. Agarose gel (2%) visualization of diagnostic polymerase chain reaction products of four *Cryptosporidium* genotypes. Lane S, standard 100-bp ladder; lane 1, patient 53, zoonotic genotype 2; lane 2, patient 119, *Cryptosporidium* sp. (zoonotic, canine genotype); lane 3, patient 84, *C. felis* (zoonotic, feline genotype); lane 4, patient 75, anthroponotic genotype 1.

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Cryptosporidium felis                 AAGCTCGTAG TTGGATTTCT GTTAATACCT TATATATAAT ATTTTTTTTT AAATATTATT   60
Cryptosporidium sp. (canine)          .......... .......... .......AT. .......... ....------ -..C..AT..   53
Cryptosporidium parvum genotype 1     .......... .......... .......AT. ......A... .....----G .TGA..AT..   56
C. parvum genotype 2                  .......... .......... .......AT. ......A... .....----G .TGA..AT..   56

C. felis                              ATGTAAGATT AACATAATTC ATATTTTTTA AGACTGAATT TTT----AGT TTTGATAATA  116
Cryptosporidium sp. (canine)          ..A...T... .......... .....----- --...ATT.A .------... -------...   93
C. parvum genotype 1                  ..A...T... .......... .....----- --...ATT.. ...TTTT... -------...  102
C. parvum genotype 2                  ..A...T... .......... .....----- --...AT..A ...T---... -------...   99

C. felis                              TGAAATTTTA CTTTGAGAAA ATTAGAGTGC TTAAAGCAGG CTTTTGCCTT GAATACTCCA  176
Cryptosporidium sp. (canine)          .....C.... .......... .......... .......... .......... .......AG.  153
C. parvum genotype 1                  .......... .......... .......... .......... .A.A...... ..........  162
C. parvum genotype 2                  .......... .......... .......... .......... .A.A...... ..........  159

C. felis                              GCATGGAATA ATAATA--AA AGATTTTTAT CTTTTTTTTA TTGGTTCTAA GATAAAAATA  236
Cryptosporidium sp. (canine)          ......---. ......TT.. .......... ....C..--. .......... ....G.....  208
C. parvum genotype 1                  ......---. ......TT.. .......... .......--. .......... .....G....  217
C. parvum genotype 2                  ......---. ......TT.. .......... ....C..--. .......... .....G....  214

C. felis                              ATGATTAATA GGGACAGTTG GGGGCATTTG TATTTAACAG TCAGAGGTGA TATTCTTAGA  294
Cryptosporidium sp. (canine)          .......... .......... .......... .......... .T........ A.........  268
C. parvum genotype 1                  .......... .......... .......... .......... .......... A.........  277
C. parvum genotype 2                  .......... .......... .......... .......... .......... A.........  274
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Figure 2. Alignment of the *Cryptosporidium* small subunit ribosomal DNA (SSU-rRNA) diagnostic fragments obtained with the CPBDIAGF/CPBDIAGR polymerase chain reaction primer pair for the four genotypes. Only the first 300 columns of the alignment are shown, as the remaining columns were identical for all genotypes. Gaps are shown with dashes (-), and bases identical to the base in the first row (*C. felis*) are shown with dots (.). Numbers to the right of the alignment show sequence positions for each genotype. The sequences for the SSU-rRNA diagnostic fragment of all four genotypes were submitted to GenBank and were assigned accession numbers AF087574, AF087575, AF087576, and AF087577.

Results of genotyping the 18 specimens are in Table 2. For patients 53, 124, 153, 184, 278, and 485, only a single specimen was available. For other patients (e.g., patient 554), four specimens collected during 12 months were available. The same *Cryptosporidium* genotype persisted throughout a patient's infection.

Conclusions

Using the sequence of a diagnostic fragment of SSU-rRNA, as well as two well-established genotypes of *C. parvum* (anthroponotic genotype 1 and zoonotic genotype 2), we detected two new *Cryptosporidium* genotypes. The first, in patients 84, 184, and 485, was identical to the

Table 2. Persistence of *Cryptosporidium* genotypes in patients[a]

Patient no.	No. of available specimens	Month of follow-up 1	2	3	4	5	6	7	8	9	10	11	12
53	1	B											
75	2	H	-	-	H								
84	4	F	-	F	-	F	F						
119	2	C	-	C									
124	1	H											
153	1	H											
184	1	F											
278	1	H											
485	1	F											
554	4	H	-	-	-	-	-	-	H	-	H	-	H

[a]B, *C. parvum* genotype 2 (zoonotic, bovine); C, *Cryptosporidium* sp. (zoonotic, canine); F, *C. felis* (zoonotic, feline); H, *C. parvum* genotype 1 (anthroponotic); -, specimen not available.

feline *Cryptosporidium* genotype (28), also described as *C. felis*. The second, in two specimens from patient 119, represents the newly identified *Cryptosporidium* sp. found in a sequence originating from a dog (GenBank accession number AF112576).

New taxons may be established within the genus *Cryptosporidium* on the basis of the isolate's host range together with molecular data, even though morphologic criteria are apparently lacking. We propose to use *C. felis* Iseki, 1979, instead of feline *C. parvum* genotype; we believe that the anthroponotic genotype 1 of *C. parvum* and the *Cryptosporidium* sp. (canine genotype) described here should be named as distinct species. Although the taxonomy of protists is morphology-based, the taxonomy of bacteria is being reevaluated on the basis of molecular data (35). There is no reason to use different approaches to these two kingdoms, nor is it necessary to postulate that the basis for speciation of *Cryptosporidium* remains ambiguous or that molecular data lend little support to separation of *Cryptosporidium* into distinct, valid species (26). Further, we see no evidence for the unconventional conclusions of Tzipori and Griffiths that "...genetic markers in *C. parvum* can change upon passage to a different host, possibly through a selective mechanism favoring different populations" (26) nor for those of Widmer (21) that "...genetic studies and experimental infections suggest that a selective mechanism triggered by a change in the intestinal environment might be involved in shaping the genetic make-up of *C. parvum* populations."

The finding of new *Cryptosporidium* genotypes in immunodeficient patients might suggest a unique susceptibility to infections by divergent *Cryptosporidium* species circulating in companion animals or livestock. However, recent identification of *C. felis* in a cow (36) may indicate a complex pattern of flow of different *Cryptosporidium* species in the environment. When more data on the distribution of these species become available, public health and preventive measures will need to be reevaluated, and isolates may need to be renamed to reflect their natural history. Finally, it is clear that Kinyoun carbol-fuchsin modified acid-fast stain (30) and direct immunofluorescence (31) could detect all genotypes, but this may not be true for diagnostic reagents that may be affected directly (PCR) or indirectly (Ab) by the genetic composition of the new *Cryptosporidium* isolates reported here. Thus, discovery of these new *Cryptosporidium* genotypes in human cryptosporidiosis should cause existing diagnostic tools to be reevaluated.

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Dr. Pieniazek is a microbiologist with the National Center for Infectious Diseases, CDC. His research interests include molecular reference diagnosis of parasitic diseases and molecular systematics.

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Fatal Case Due to Methicillin-Resistant *Staphylococcus aureus* Small Colony Variants in an AIDS Patient

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We describe the first known case of a fatal infection with small colony variants of methicillin-resistant *Staphylococcus aureus* in a patient with AIDS. Recovered from three blood cultures as well as from a deep hip abscess, these variants may have resulted from long-term antimicrobial therapy with trimethoprim/sulfamethoxazole for prophylaxis of *Pneumocystis carinii* pneumonia.

Staphylococcus aureus causes acute and often fatal infections. Small colony variants (SCVs), which are subpopulations of *S. aureus,* are implicated in persistent and recurrent infections (in particular osteomyelitis, septic arthritis, respiratory tract infections in patients with cystic fibrosis, and deep-seated abscesses) (1-4). These phenotypic variants produce small, slow-growing, nonpigmented, nonhemolytic colonies on routine culture media, making correct identification difficult for clinical laboratories. Biochemical characterization of these variants suggests that they are deficient in electron transport activity (5).

We report a fatal case of a persistent deep-seated hip abscess due to methicillin-resistant *S. aureus* SCVs that led to osteomyelitis and bloodstream infection in a patient with AIDS.

Case Report

A 36-year-old man with AIDS came to the Cologne University Hospital, Cologne, Germany, in June 1997 with fever and progressive pain (of 6 weeks duration) in his right hip. HIV infection had been diagnosed in 1986. In 1994, his CD4 cell count was 250/µL, and oral zidovudine therapy was started. His medical history included *Pneumocystis carinii* pneumonia, pulmonary tuberculosis, and recurrent oral thrush; his medication included zidovudine, lamivudine, fluconazole, and trimethoprim/sulfamethoxazole. In September 1996, he was in a traffic accident and had severe cerebral trauma resulting in spastic hemiparesis with occasional seizures. After an intramuscular injection 2 months before admission, pus was surgically drained to treat recurrent abscesses of his right hip. Specimens for culture were not obtained.

Physical examination found limited mobility of his right thigh and a tender, nondraining scar at the site of surgical drainage. Neither warmth nor swelling was observed over his right hip. Vital signs were temperature, 38.2°C; respiration rate, 28; and heart rate, 108. He was awake and alert and had spastic paresis in his right arm.

Laboratory studies performed on admission showed hemoglobin, 10.8 g/dL; leukocyte count, 3,000/µL with a normal differential; CD4 cell count, 20/µL; platelet count, 131,000/µL; C-reactive protein, 184 mg/L; and alkaline phosphatase, 1490 U/L. Radiographs of the chest and a plain film of the pelvis were normal. A triple-phase bone scan showed an area of minor tracer accumulation in the acetabulum region of the right hip. Blood cultures were drawn, but antimicrobial therapy was withheld until culture results became available.

On hospital day 2, one of two blood cultures drawn on admission yielded nonhemolytic staphylococci that were clumping factor–negative. The organisms were initially misidentified as coagulase-negative staphylococci and were considered contaminants.

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Empiric antistaphylococcal therapy with clindamycin (600 mg q8hr) was instituted. On hospital day 4, two sets of blood cultures obtained on hospital day 2 yielded phenotypically identical organisms, which on the basis of a positive tube coagulase test were identified as oxacillin-resistant *S. aureus*. The colony morphology was suggestive of an SCV of *S. aureus*. The patient was started on parenteral vancomycin treatment (1 g q12hr). However, his condition deteriorated rapidly, and he died of refractory septic shock 6 days after admission.

Autopsy showed a large (12 x 10 x 8 cm), deep-seated abscess of the right hip and osteomyelitis of the ischial tuberosity. Both SCVs and typical large colony forms of *S. aureus* were cultured from postmortem specimens of the abscess and the bone.

Findings

S. aureus SCVs were recovered from one of two blood culture sets obtained on admission and from two of four blood culture sets obtained on hospital day 2. Growth was not detected until the blood culture bottles had been incubated 24 hours. *S. aureus* with a normal phenotype was recovered from nose and throat specimens but not from blood cultures, whereas both SCVs and typical *S. aureus* phenotypes were isolated from the deep hip abscess (Figure 1) before death, as well as from a postmortem specimen. All isolates were clumping factor–negative but showed a delayed positive reaction in the tube-coagulase test at 24 hours. The results of the ID 32 staph test did not unambiguously identify SCVs as *S. aureus* because the tests for urease and trehalose were negative. Both the *nuc* gene and the *coa* gene were identified by polymerase chain reaction (PCR) amplification. Methicillin resistance was confirmed for both small and large colony forms by PCR amplification of the *mecA* gene.

When cultured without supplementation, all SCVs were nonpigmented and nonhemolytic. Supplementation with hemin, thymidine, or menadione identified two SCVs showing thymidine auxotrophy and a combined thymidine and menadione auxotrophy, respectively. All SCVs were stable on repeated subculturing.

Epidemiologic typing by PCR analysis of inter-IS256 spacer length polymorphisms (Figure 2) and pulsed-field gel electrophoresis of genomic DNA (data not shown) showed identical banding patterns for both SCVs and large colony forms, which indicates that the phenotypically different *S. aureus* isolates represented a single strain. Antimicrobial susceptibility testing was performed by microbroth dilution, according to the National Committee for Clinical Laboratory Standards guidelines. Susceptibility to trimethoprim/sulfamethoxazole was tested with Etest (AB Biodisk, Solna, Sweden). In contrast to current standards, the MICs for SCVs were determined after 48 hours of incubation at 35°C. Susceptibility testing showed that all *S. aureus* isolates were resistant to penicillin (MIC, >8 µg/mL), ampicillin (MIC, >32 µg/mL), oxacillin (MIC, >8 µg/mL), erythromycin (MIC, >32 µg/mL), clindamycin (MIC, >32 µg/mL), ciprofloxacin (MIC, >8 µg/mL), gentamicin (MIC, >500 µg/mL), and trimethoprim/sulfamethoxazole (MIC, >32 µg/mL) and susceptible to vancomycin

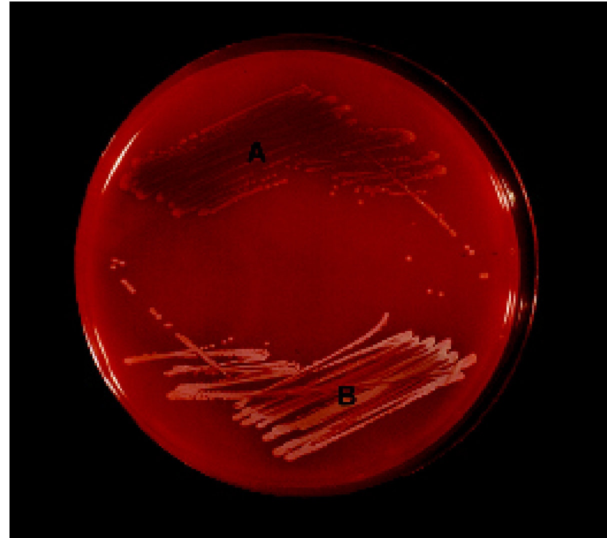

Figure 1. *Staphylococcus aureus* small colony variants (A) and *S. aureus* with a normal phenotype (B) cultured on sheep blood agar after 24 hours of incubation at 35°C. Staphylococci were identified by conventional methods (6) and with the ID 32 Staph system (bioMérieux, Marcy-L'Etoile, France) following the instructions of the manufacturer. The tube-coagulase test was read after 24 hours. *S. aureus* isolates were characterized as small colony variants as described before (7-8). Auxotrophic requirements were evaluated with 10-µg hemin disks, 1.5-µg menadione disks, and 1.5-µg thymidine disks on Mueller-Hinton agar and on chemically defined medium (CDM) agar as well as on CDM agar supplemented with 1 µg/mL hemin, 100 µg/mL thymidine, and 1 µg/mL menadione, respectively.

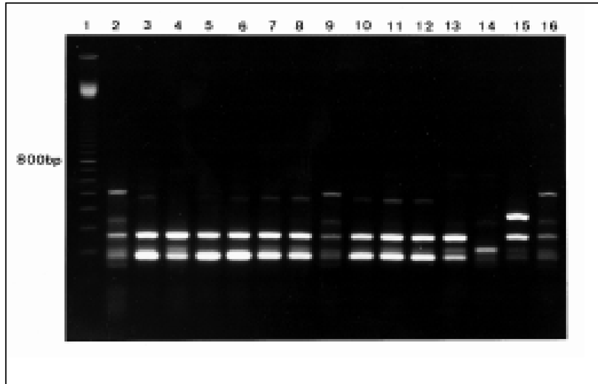

Figure 2. Fingerprint patterns obtained for *Staphylococcus aureus* small colony variants (lanes 3-5, bloodculture isolates; lanes 6 and 7, isolates from hip abscess; lane 8, postmortem specimen) and *S. aureus* isolates with a normal phenotype (lanes 10 and 11, isolates from nose and throat; lanes 12 and 13, isolates from hip abscess and postmortem specimen) after polymerase chain reaction (PCR) analysis of inter-IS256 spacer length showing identical strains. Lane 1, 100-bp ladder; lanes 2, 9, and 16, methicillin-resistant *S. aureus (*MRSA) reference strain; lanes 14 and 15, epidemiologically unrelated MRSA strains. Strain relatedness of all isolates with different colony morphologies and from different sources was analyzed by PCR analysis of inter-IS256 spacer length polymorphisms (9) and pulsed-field gel electrophoresis after *Sma*I restriction (8). Minor modifications included the use of brain heart infusion broth instead of trypticase soy broth to obtain sufficient growth of *S. aureus* small colony variants.

(MICs, 1-2 µg/mL), teicoplanin (MICs, 0.5-1 µg/mL), and quinupristin/dalfopristin (MICs, 0.5-1 µg/mL). No differences in MICs were observed between *S. aureus* SCVs and *S. aureus* isolates with normal phenotype.

To our knowledge, this case represents the first of a serious *S. aureus* infection in an AIDS patient in which all blood cultures yielded SCVs. The SCVs' unusual morphologic appearance and slow growth delayed the correct identification of these organisms as *S. aureus*. The empiric antimicrobial regimen in our patient did not include a glycopeptide, because of the low rate of methicillin resistance in community-acquired *S. aureus* infection in Germany. Appropriate antistaphylococcal therapy was, therefore, not started until hospital day 4. Delayed antimicrobial therapy on day 4 rather than on day 2 may have contributed to the patient's death.

Proctor and colleagues recently reported five cases in which SCVs of *S. aureus* were implicated in persistent and relapsing infections. They identified only a single case reported in the previous 17 years and ascribed this to insufficient ability of laboratories to identify these organisms (8). In most cases, patients had received antibiotics. Aminoglycoside treatment may have selected for *S. aureus* SCVs (10), and in cases of osteomyelitis or deep-seated abscesses, persistence of these variants in the intracellular milieu may have permitted evasion of host defenses and allowed for the development of resistance to antimicrobial therapy (7,11). Von Eiff and colleagues recently reported four cases of chronic osteomyelitis due to SCVs of *S. aureus* in patients who had received gentamicin beads as an adjunct to surgical therapy for osteomyelitis (2). Kahl et al. described persistent infection with *S. aureus* SCVs in patients with cystic fibrosis (4). All these patients had received long-term trimethoprim/sulfamethoxazole prophylaxis. It may be tempting to speculate that administration of trimethoprim/sulfamethoxazole for prophylaxis against *P. carinii* pneumonia may have selected for SCVs within the patient's large hip abscess. Further prospective studies are needed to assess the role of *S. aureus* SCVs in HIV-infected patients on long-term antimicrobial therapy.

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Application of Data Mining to Intensive Care Unit Microbiologic Data[1]

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We describe refinements to and new experimental applications of the Data Mining Surveillance System (DMSS), which uses a large electronic health-care database for monitoring emerging infections and antimicrobial resistance. For example, information from DMSS can indicate potentially important shifts in infection and antimicrobial resistance patterns in the intensive care units of a single health-care facility.

We have defined a new exploratory data mining process for automatically identifying new, unexpected, and potentially interesting patterns in hospital infection control and public health surveillance data. This process, and the system based on it, Data Mining Surveillance System (DMSS), use association rules to represent outcomes and association rule confidences to monitor changes in the incidence of those outcomes over time. Through experiments with infection control data from the University of Alabama at Birmingham Hospital, we have demonstrated that DMSS can identify potentially interesting and previously unknown patterns. Future work on prospective clinical studies to determine the usefulness of DMSS in hospital infection control is needed, as is improved event presentation for the user and strategies for handling larger datasets.

The statistical strategies developed for automatically detecting temporal patterns in surveillance data require that analysts explicitly define outcomes of interest before surveillance begins. The Data Mining Surveillance System (DMSS), on the other hand, is not constrained to monitoring changes in user-defined outcomes. In DMSS, complex outcomes are represented by association rules, and outcome incidence is captured monthly.

An early version of DMSS, along with association rules and early experiments with a single organism, has been described (1). We briefly describe a newer version of DMSS and experimental results obtained by using it to analyze 1 year's data from intensive care units (ICUs) at the University of Alabama at Birmingham Hospital.

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DMMS uses the following definitions. An itemset is a subset of the set of all items. The support of an itemset x, sup (x), is the number of records that contain x. If sup $(x) \geq$ FSST, where FSST is the frequent set support threshold (FSST), then x is a frequent set. An association rule, A $\Rightarrow$ B, where A and B are frequent sets and $A \cap B = \emptyset$, is a statement about how often the items of B are found with the items of A. The incidence proportion of A $\Rightarrow$ B, denoted ip(A $\Rightarrow$ B), is equal to sup$(A \cup B)$/sup(A). The precondition support of association rule A $\Rightarrow$ B is sup(A). The incidence proportion of an association rule A $\Rightarrow$ B in data partition p_i describes the incidence of the outcome, B, in the group, A, during time t_i. A series of incidence proportions for A $\Rightarrow$ B from partitions p_1, p_2, ..., p_n describes the incidence of the outcome B in group A from t_1 through t_n. Therefore, by analyzing the series of incidence proportions of an association rule A $\Rightarrow$ B, it should be possible to detect important shifts or trends in the incidence of B in A over time. In this way, surveillance of B in A is possible.

Bacterial susceptibility and related demographic data of patients in the University of Alabama at Birmingham Hospital ICUs (medical, surgical [SICU], cardiac, neurologic [NICU]) during 1997 were extracted from the PathNet laboratory information system. Each record describes a single isolate and contains the following data elements: date of admission, date

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of sample collection, date of results reported, source of isolate (e.g., sputum, blood), organism isolated, organism Gram stain and morphologic features, patient's location in the hospital, and resistant (R), intermediate (I), or susceptible (S) test results to relevant antibiotics, according to the National Committee for Clinical Laboratory Standards MIC breakpoints (2).

Duplicate records were removed so that for each patient, no more than one isolate per organism per month was included. In each remaining record, certain antimicrobial drug items were removed (only drugs to which the organism is historically susceptible at least 50% of the time remained). Additionally, items of the form S~Antimicrobial were removed so that only I~Antimicrobial and R~Antimicrobial items remained. Finally, data were divided into 1-month partitions ($p_1 \ldots p_n$) before analysis. For each partition p_i, all frequent sets with support of at least 3 (FSST >2) and association rules with precondition support greater than 5 were generated. Both the frequent set discovery and association rule-generating algorithms are beyond the scope of this review (3).

Each generated association rule must pass a set of rule templates that describe families of interesting and uninteresting rules. Each template is a construct of the form $be_1 \Rightarrow be_2$, where be_1 and be_2 are Boolean expressions over items and attributes. Association rule $A \Rightarrow B$ satisfies rule template $be_1 \Rightarrow be_2$ if A satisfies be_1 and B satisfies be_2. Two types of association rule templates are used: include templates and exclude templates. An association rule $A \Rightarrow B$ passes a set of rule templates if $A \Rightarrow B$ satisfies at least one include template in the set and does not satisfy any exclude template in the set.

Rule templates are handcrafted by domain experts to eliminate inherently uninteresting or nonsense rules. This is accomplished through iterative experiments with representative data by initially using few templates and then creating and modifying templates on the basis of pattern review.

History is a database that holds association rules and their incidence proportions for different data partitions. In DMSS, the user specifies a set of rule templates that contains any number of inclusive and restrictive templates (Table 1). Only association rules that pass the rule templates are included in the history. To establish a baseline for an association rule, the incidence proportions of the rule for the three previous partitions are obtained and stored in the history. Once stored in the history, a rule is updated for each new partition regardless of whether or not it is generated in the partition. Therefore, for every association rule, the history contains an up-to-date time-series of incidence proportions.

By analyzing information stored in the history, DMSS generates alerts that describe an extreme change in the incidence of an outcome B in a group A over time. For example, Table 2 describes the incidence of *Acinetobacter baumannii* in a nosocomial tracheal aspirate and in SICU isolates over the past six partitions. Clearly, a shift in incidence occurs between the first 4 months and the most recent 2 months of the series. If we call months 1, 2, 3, and 4 the past window, w_p, and months 5 and 6 the current

Table 1. Templates used to filter association rules

Template type	Left (be_1)	Right (be_2)	Explanation
Exclude	(R~Antibiotic)	(Anything)	Want antibiotic sensitivity info on the right only.
Exclude	(Anything)	(Source)	Source of infection is not an outcome. Therefore, exclude all rules with a source on the right.
Exclude	(NS OR Org OR GrMP)	(NS OR Org OR GrMP	NS, Org, and GrMp are more informative if kept together in either a group or an outcome.
Exclude	(Loc)	(Org OR GrMp) AND (R~Antibiotic)	If the left contains location, then exclude rules that have Org and R~Antibiotic or GrMp and R~Antibiotic.
Include	(Org OR Loc)	(R~Antibiotic OR GrMp OR Org) AND Not(Loc)	Include rules whose groups are Org- or Loc-specific and whose outcomes are Antibiotic- or GrMp-specific.

be_1 and be_2, Boolean expressions; R, resistant; NS, nosocomial; OR, "or"; Org, organism; GrMp, Gram stain and morphology; Loc, Location.

Table 2. A sample event generated by the Data Mining Surveillance System

Association rule			p_{c-5}[a]	p_{c-4}	p_{c-3}	p_{c-2}	p_{c-1}	p_c
(nosocomial, SICU[b], tracheal aspirate}	==>	{*Acinetobacter baumannii*}	0/11	0/10	0/9	0/13	**2/9**	**3/9**
				w_p[c]			w_c	

[a]P_c, current pair.
[b]SICU, surgical intensive care unit.
[c]w_p, past window; w_c, current window.

window, w_c, we can ask if there is an extreme change in the incidence between w_p and w_c. We compute the cumulative incidence proportion for w_p (0/43) and for w_c (5/18) and compare the two by a statistical test of two proportions. To generate an alert for an association rule r, DMSS first constructs a current window (w_c) and a past window (w_p) on the series of incidence proportions of r ($w_c[r,0]$, $w_p[r,0]$ from the algorithm in the Figure). Second, it computes the cumulative incidence proportion for each window. Third, it compares the two cumulative incidence proportions by a test of two proportions. Finally, if the difference between the proportions is statistically extreme ($p \leq \alpha = 0.01$), it generates an alert. The value of α is user-defined and rather arbitrary. If an alert is not generated, the next set of current and past windows is formed ($w_c[r,1]$, $w_p[r,1]$ from the algorithm in the Figure), and the cumulative incidence proportions are compared. Window pairs are generated for the same association rule until an alert is generated or no more window pairs remain to be formed. DMSS generates all alerts by executing the procedure described on every association rule in the history.

Current and past window pairs are generated by the algorithm in the Figure. If n is the number of incidence proportions in the history for a given rule, (w_c:w_p) pairs are generated for that rule in the following order: (p_c:[p_{c-1},p_{c-2}]}),...,(p_c:[p_{c-1},...,p_{c-n}]]),([p_c,p_{c-1}],[p_{c-2},p_{c-3}]}),([p_c,p_{c-1}]},[p_{c-2},p_{c-3},p_{c-4}]),([p_c,p_{c-1}],[p_{c-2},p_{c-3},p_{c-4},...,p_{c-n}]), ([p_c,p_{c-1},p_{c-2}],[p_{c-3},p_{c-4},p_{c-5}]}),([p_c,p_{c-1},p_{c-2}]},[p_{c-3},p_{c-4},p_{c-5},p_{c-6}]}),...,([p_c,p_{c-1},p_{c-2}]},[p_{c-3},p_{c-4},p_{c-5},p_{c-6},...,p_{c-n}]). For each pair, w_p must be at least as large as w_c.

The total number of events was reduced from 251, by including all rules, to 36, by using the templates in Table 1; thus, classes of inherently uninteresting rules were eliminated. A retrospective look at the 155 events eliminated by the rule templates showed that they were uninformative. Therefore, the introduction of templates resulted in a more focused presentation of DMSS output.

Of the 36 events, 18 were judged potentially interesting. Table 3 contains several representative events, one per row. Each row contains the association rule, the incidence proportions in w_c (bold), and the incidence proportions in w_p (nonbold). For example, event 1 in Table 3 describes an increase in the number of *Staphylococcus aureus* resistant to oxacillin, clindamycin, and erythromycin isolated from tracheal aspirates in the fourth partition, and compared with those isolated in the 2nd and 3rd partitions. Of the events identified by DMSS, only the NICU and SICU had events that were location-specific (Table 3), while eight events were not.

The events identified by DMSS must be investigated by domain experts to determine

i=0; k=0
while (p_{c-2i-1} exists for *r*){
    j=0
    while ($p_{c-2i-1-j}$ exists for *r*){

$$w_c[r,k] = \bigcup_{n=0}^{i} p_{c-n}$$

$$w_p[r,k] = \bigcup_{n=0}^{i+j} p_{c-n-i-n-1}$$

        j++; k++
    }
    i++
}

Figure. Algorithm used to construct current and past windows for association rule r.

Table 3. Representative events identified and considered of potential interest

Left Denominator		Right Numerator	Partition 1	2	3	4	5	6	7	Interpretation
Staphylococcus aureus Source TRACHASP[c]	==>	R~Oxacillin[a,b] R~Clindamycin R~Erythromycin		0/10	0/8	**7/14**				Increase in the incidence of oxacillin (ORSA), clindamycin, and erythromycin resistance in all *S. aureus* isolated from tracheal aspirates.
NSNoso[d]	==>	R~Ceftazidime				3/88	**11/70**			Increase in incidence of ceftazidime resistance in all nosocomial isolates.
NP_GNR[e]	==>	R~Piperacillin				0/17	**6/14**			Increase in the LocSICU incidence of piperacillin resistance in non-pseudomonas gram-negative bacilli isolated from NSNoso.
NP_GNR	==>	R~Piperacillin			1/12	0/14	**4/11**	**4/8**		Increase in the LocSICU[f] incidence of piperacillin resistance in non-pseudomonas, nosocomial, gram-negative bacilli from the SICU.
NSNoso LocNICU[g]	==>	*S. aureus*	3/26	3/26	2/28	**6/27**	**5/20**	**3/11**		Increase in the incidence of nosocomial *S. aureus* in nosocomial isolates from the NICU.

[a]R, resistant.
[b]Oxacillin, resistance implies resistance to amoxycillin/clavulanic acid, cephalothin, and cefazolin.
[c]SourceTRACHASP, tracheal aspirates.
[d]NSNoso, nosocomial (3 days from admission).
[e]NP_GNR, non-pseudomonas gram-negative rod.
[f]LocSICU, location, surgical intensive care unit (SICU).
[g]LocNICU, location, neonatal intensive care unit (NICU).

their actual importance. In this example, the data burden was small since in a prospective analysis only a few events would be presented to the user each month, thus allowing for the investigation of each event.

We believe that this approach to surveillance will allow hospital infection control programs to focus their limited resources on issues of probable significance. We also believe that this approach is a step toward the public health surveillance system described by Dean, Fagan, and Panter-Conner (4).

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Sentinel Surveillance for Enterovirus 71, Taiwan, 1998

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Outbreaks of enterovirus 71 have been reported around the world since 1969. The most recent outbreak occurred in Taiwan during April-July 1998. This hand, foot, and mouth disease epidemic was detected by a sentinel surveillance system in April at the beginning of the outbreak, and the public was alerted.

Enterovirus type 71 (EV71), one of the etiologic agents of epidemic hand, foot, and mouth disease (HFMD), has been associated with febrile rash illness, aseptic meningitis, encephalitis, and a syndrome of acute flaccid paralysis similar to that caused by poliovirus (1,2). EV71 was identified in 1969 in the United States, when it was isolated from the feces of an infant with encephalitis in California. By 1998, many EV71 outbreaks had been reported around the world. In addition to the outbreak in California in which one death was reported, four other outbreaks resulted in many fatal cases involving clinical deterioration and death in young children (Bulgaria, May-September 1975; Hungary, 1978; Malaysia, April-June 1997; Taiwan, April-August 1998) (3-5). To the best of our knowledge, the outbreak in Taiwan marked the first time that an EV71 outbreak was detected by a surveillance system, which alerted the public about the epidemic of HFMD. We describe how the EV71 outbreak was reported by a sentinel surveillance system established in July 1989 by Disease Surveillance and Quarantine Service (originally National Quarantine Services), Ministry of Health, Taiwan.

In this sentinel surveillance system, public health officers contact local and regional physicians weekly to actively collect disease information. On the basis of information collected, disease incidence trends are predicted, and, if necessary, the public is warned during the earliest stage of disease outbreaks. Mumps, varicella, diarrhea, and upper-tract respiratory infection are among the diseases subject to routine surveillance. For convenience, we have established two channels of data collection: telephone interviews and report cards mailed by physicians. Approximately 850 physicians (fewer than one tenth of the physicians) from every county in Taiwan participate in the system: 258 in the northern region, 211 in the central region, 296 in the southern region, and 85 in the eastern region (Table); most of these are pediatricians, general practitioners, and family physicians. Only a few are ear, nose, and throat specialists.

When an epidemic of fatal myocarditis was reported in Sarawak, Malaysia, in 1997, we started to collect information regarding HFMD and vesicular pharyngitis (herpangina) through

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Table. Physician distribution, Sentinel Surveillance System, Taiwan

Region of Taiwan	Interviews	No. cards mailed by physicians	Total no. physicians
Northern	109	149	258
Central	27	184	211
Southern	175	121	296
Eastern	13	72	85
Total	324	526	850

the sentinel surveillance system. Beginning in March 1998, some physicians reported a notable increase of cases of HFMD, vesicular pharyngitis (herpangina), and vesicular stomatitis exanthem. Furthermore, a dramatic upsurge of HFMD was seen in children in outpatient settings at the end of April 1998 (Figure 1).

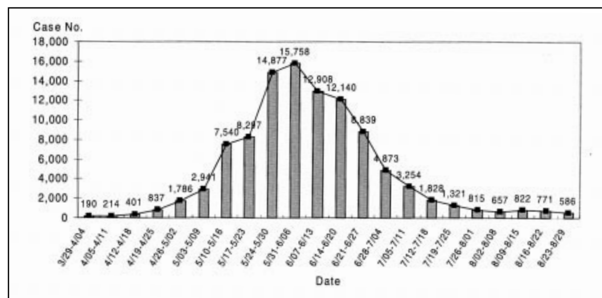

Figure 1. Total cases of hand, foot, and mouth disease and herpangina reported from sentinel physicians in Taiwan, March 19 to August 29, 1998.

Surveillance data provided the basis for immediate public health action. When the number of weekly reported cases increased twofold at the end of April, the public was informed about the epidemic of HFMD and herpangina and the threat of enterovirus infection (May 12). Measures for preventing the spread of infection (e.g., practicing good hygiene at all times, confining infected children at home, avoiding contact with infected children) were advised. However, the number of reported cases still dramatically increased to approximately 3,000 in the following week. Although some errors in reporting might have occurred, we are confident that the reporting quality was adequate. The sentinel physicians, who have voluntarily cooperated with us for 10 years, used a clinical case definition of HFMD/herpangina we created when the surveillance started.

Monitoring incidence of the fatal and most severe cases of HFMD appeared critical; therefore, another report system, designed for monitoring severe and fatal cases, was established (May 29) to enroll all well-defined severe and fatal cases in 597 hospitals and medical centers. This new system established a network of various public health agencies, general and regional hospitals, and medical centers. The difference between the two systems was that, while the sentinel surveillance system was physician-based, the new system was hospital-based. Severe and fatal cases were defined as HFMD with complications, including aseptic meningitis, encephalitis, myocarditis, acute flaccid paralysis, rapidly deteriorating clinical course, and death. Both surveillance systems worked simultaneously from June 1998 onward. We found that the trend peaked and declined earlier in the sentinel system than in the hospital-based surveillance system (Figure 1, 2).

A case-control study was implemented, and enterovirus isolation data were reviewed. Several enterovirus isolations, from patients with severe and fatal cases, were in stool specimens, throat secretions, cerebrospinal fluid, blood, and central nervous system tissue, including EV71, Coxsackie, and ECHO. Additional studies comparing rates of EV71 isolation in different years are in progress and will be reported separately. Most isolated viruses were EV71 from all specimens in this epidemic. The epidemiologic, clinical, and virologic evidence suggests an association between EV71 infection and this epidemic of HFMD. However, the causes of the severe cases and deaths in Taiwan are yet to be defined (5).

A physician-based sentinel surveillance system can play an important role in preventing emerging infectious diseases. Even though the data collected may be rough, a sentinel surveillance system can provide necessary information for monitoring communicable diseases, guiding further investigation, and evaluating control measures, as well as early warning for epidemics and rationale for public

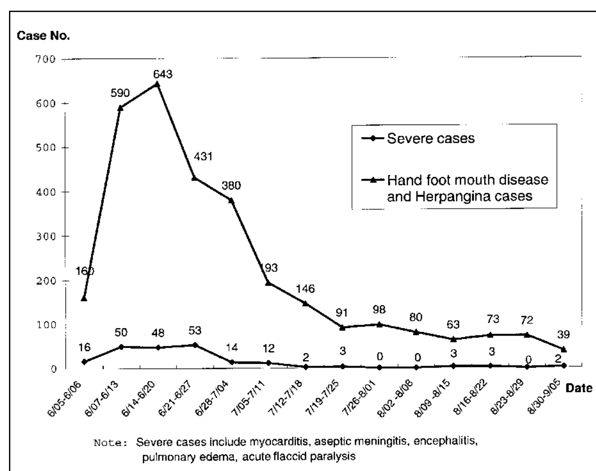

Figure 2. Number of hospitalizations and severe cases of hand, foot, and mouth disease and herpangina in Taiwan, June-August, 1998.

health intervention. Early detection of communicable diseases and immediate public health intervention can curtail the number of illnesses and deaths and reduce negative effects on international travel and trade (6).

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Chlorine Inactivation of *Escherichia coli* O157:H7

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We analyzed isolates of *Escherichia coli* O157:H7 (which has recently caused waterborne outbreaks) and wild-type *E. coli* to determine their sensitivity to chlorination. Both pathogenic and nonpathogenic strains were significantly reduced within 1 minute of exposure to free chlorine. Results indicate that chlorine levels typically maintained in water systems are sufficient to inactivate these organisms.

Escherichia coli O157:H7 is becoming increasingly recognized as a waterborne pathogen. Two recent outbreaks during summer 1998, one involving a drinking water supply in Wyoming (1) and another involving recreational water exposure at a water park in Georgia (2), have underscored the role of water in transmission. Contaminated drinking water (3,4) and recreational water have been associated with outbreaks of hemorrhagic colitis caused by *E. coli* O157:H7 (5-7). Chlorination of water is one of the primary public health measures used to ensure that both potable water and water used in recreational settings are free of microbial pathogens. Our study was undertaken to determine the chlorine resistance of *E. coli* O157:H7 and compare this resistance with that of wild-type *E. coli*.

Seven strains of *E. coli* O157:H7, isolated from cattle from geographically distinct areas (Florida, Idaho, Illinois, Missouri, Texas, Washington, and Wisconsin), were obtained from the U.S. Department of Agriculture (D. Miller, Ames, IA). The isolates exhibited the characteristic phenotypic traits: sorbitol-negative, ß-glucuronidase–negative, lactose-positive, indole-positive, and positive for glutamate decarboxylase (8). All enterohemorrhagic isolates were active toxin producers, as determined by in vitro enzyme immunoassay (Meridian Diagnostics, Inc., Cincinnati, OH). These cattle isolates were chosen as representative strains that might contaminate water supplies after surface run-off from pastures and fields. Four wild-type *E. coli* isolates from cattle manure from a local dairy farm (Ohio) were characterized by biochemical test kits (bioMerieux Vitek, Hazelwood, MO). All bacterial cultures used in the disinfection experiments were grown for 18 to 20 hours at 35ºC in brain heart infusion broth, concentrated by centrifugation, and washed three times in phosphate buffer (9) before testing.

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The results of the disinfection experiments, including the rates of inactivation, are shown in the Table. Initial levels for all isolates were 5.52 to 5.79 $\log_{10}$ CFU/ml. The mean chlorine levels at each exposure time were 1.1 mg/L free chlorine and 1.2 mg/L total chlorine. For both the pathogenic and the wild-type strains, exposure to these levels of chlorine for 1 minute reduced the viable populations by approximately four orders of magnitude. The inactivation rates and corresponding correlation coefficient (r^2) values are listed in the Table. Little difference was observed in the rates of inactivation for the pathogenic and wild-type organisms.

These results indicate that the *E. coli* O157:H7 isolates used in this study were sensitive to chlorination and were similar in resistance to that of wild-type *E. coli* isolates. The biocidal activity of chlorine decreases with decreasing temperature (not done in this study). The 5ºC temperature we used represents a worst-case condition for both ground water or winter surface-water temperature. A survey of disinfection practices in the United States found that water utilities maintain a median chlorine residual of 1.1 mg/L and a median exposure time of 45 minutes before the point of first use in the distribution system (10). At this level of

Table. Chlorine inactivation of *Escherichia coli* O157:H7 and wild-type *E. coli* [a]

	Log$_{10}$CFU/ml					
	Initial	After exposure time of			Inactivation	
Isolate	inoculum	30 sec	60 sec	120 sec	rate (sec^{-1})	r^2
E. coli O157:H7						
N009-6-1	5.63	2.60	1.88	0.82	-2.96	0.82
N6001-8-10	5.78	2.52	1.44	0.72	-3.06	0.68
N6021-5-1	5.78	2.54	1.52	0.66	-3.06	0.54
N60049-26-1	5.68	2.35	1.40	0.54	-3.00	0.86
N6059-7-2	5.72	2.42	1.74	0.86	-3.02	0.72
N6104-5-9	5.62	2.40	1.69	0.72	-2.96	0.89
N6114-7-2	5.63	2.52	1.66	0.89	-2.96	0.82
Mean	5.69	2.48	1.62	0.74	-2.93	0.82
E. coli (wild type)						
A	5.53	2.66	1.80	1.52	-2.51	0.61
B	5.79	2.60	1.48	0.81	-2.68	0.60
C	5.68	2.48	0.92	0.84	-2.61	0.61
D	5.52	2.34	0.95	0.39	-2.50	0.61
Mean	5.63	2.52	1.28	0.89	-2.93	0.71

[a]In chlorine demand-free chlorinated (CDF) buffer, 5°C, pH 7.0, 1.1 mg/L free chlorine, 1.2 mg/L total chlorine. Duplicate chlorine inactivation experiments were conducted in CDF buffer at pH 7.0. All experiments were conducted at 5°C in a recirculating, refrigerated water bath. The chlorinated buffer was prepared by the addition of reagent-grade sodium hypochlorite (Fisher Scientific, Fair Lawn, NJ). Reaction vessels were continuously mixed (250 rpm) by using an overhead stirring apparatus equipped with sterile stainless steel paddles. Chlorine concentrations were determined by the N,N-dimethyl-*p*-phenylenediamine colorimetric method (9). Samples were removed from the reaction vessels at the desired exposure times, and the chlorine was immediately neutralized by the addition of 0.5 ml of 10% (wt/vol) sodium thiosulphate. Vessels containing CDF buffer without chlorine served as controls for determining unexposed concentrations of the bacteria. Initial levels and the number of survivors after chlorine exposure were determined by the membrane filtration procedure using mT7 agar incubated for 22 to 24 hours at 35°C. This medium was chosen because of its ability to recover oxidant-stressed organisms (9). Levels of bacteria were determined by duplicate filtrations of appropriate dilutions for each exposure time.The log$_{10}$-transformed data were used to determine the levels of inactivation for each isolate. The means for the inactivation data for the *E. coli* O157:H7 isolates and for the wild-type *E. coli* isolates at each exposure time were used to compare the inactivation rates between the pathogenic and the wild-type organisms. The following first order model was used to describe the inactivation rate: $y = y_{10}10^{-at}$, where t = time in seconds, y = CFU/ml at any time t, y_{10} = CFU/ml at time zero, and a = the inactivation rate in sec^{-1}. The log transformation of this equation was used to calculate the inactivation rate. A regression analysis using least squares was conducted for experiments with each individual isolate and for the mean values for each of the two types of isolates (serotype O157 and wild-type) to determine the inactivation rates ("a" values).

chlorination, *E. coli* O157:H7 is unlikely to survive conventional water treatment practices in the United States. *E. coli* O157:H7 survives at a similar rate to that of wild-type *E. coli* in nondisinfected drinking water (11). Survival patterns and sensitivity to chlorination previously observed for the strains used in this study suggest that wild-type *E. coli* could serve as an adequate indicator organism for fecal contamination of water. Using wild-type *E. coli* to indicate *E. coli* O157:H7 would be useful because most analytical procedures for detecting *E. coli* in drinking water (e.g., assays for lactose fermentation at 44°C to 45°C or production of the enzyme ß-glucuronidase) cannot detect pathogenic *E. coli* O157:H7 strains (8).

Although chlorination appears to adequately control this pathogen, not all municipal water supplies use chlorine disinfection. In addition, chlorine residual can dissipate under adverse conditions, and exposure to sunlight or organic chlorine-demand substances can greatly diminish chlorine levels. Protection of organisms associated with particulate matter, such as fecal material, can also readily decrease the biocidal activity of chlorine. These considerations are particularly important in determining the efficacy of chlorination in a recreational water setting. The results of this study indicate that the isolates studied were sensitive to chlorination. Evaluation of other isolates under differing environmental conditions would be worthy of further consideration.

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Fulminant Meningococcal Supraglottitis: An Emerging Infectious Syndrome?

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We report a case of fulminant supraglottitis with dramatic external cervical swelling due to associated cellulitis. Blood cultures were positive for *Neisseria meningitidis.* The patient recovered completely after emergency fiberoptic intubation and appropriate antibiotic therapy. We summarize five other cases of meningococcal supraglottitis, all reported since 1995, and discuss possible pathophysiologic mechanisms.

Neisseria meningitidis, a gram-negative diplococcus, can cause a broad spectrum of clinical manifestations including acute meningitis, meningococcemia, occult bacteremia, meningoencephalitis, pneumonia, conjunctivitis, dermatitis-arthritis syndrome, and urethritis (1). We describe a rare case of meningococcal supraglottitis (2-6) further complicated by cervical cellulitis. Reports of five other cases suggest an emerging clinical syndrome due to this pathogen.

Case Report

In January 1998, a 44-year-old woman became ill with rhinitis and sore throat, which progressed to dysphagia, dyspnea, and neck swelling during the night, requiring her to sleep sitting upright. The following morning, she went to a community hospital emergency room.

The patient was alert but appeared toxic and had inspiratory stridor and a muffled voice. She had routine dental cleaning 1 week before onset of symptoms. She did not have a toothache and said she did not use tobacco or alcohol. She had had no history of splenectomy or immune deficiency. Her temperature was 38.1°C, pulse 138, cuff blood pressure 120/74 mm Hg, and respiratory rate 24. Room air pulse oximetry was 94%. The tongue was large, but not edematous, and there was neither drooling nor sublingual swelling. The soft palate and posterior pharynx, seen only with difficulty, were diffusely swollen, protruding anteriorly, and covered with exudate. Massive external swelling, tenderness, and erythema of the anterior neck extended from the chin caudad to the midsternum, obliterating all cervical landmarks. The patient did not have meningismus, crepitus, or jugular venous distention. The lungs and heart were normal. No rash was present.

A portable lateral radiograph of the neck showed diffuse soft tissue cervical and epiglottic swelling with a classic "thumb sign." White blood count was 21.9 $\times 10^9$/L with 0.70 polymorphonuclear leukocytes and 0.17 bands. Platelet count was 242 $\times 10^9$/L. Hematocrit, electrolytes, glucose, and urea nitrogen were normal. The patient was treated with oxygen and nebulized epinephrine, ceftriaxone 1.0 gram intravenously (i.v.), and clindamycin 600 mg i.v. She was taken to the operating room, where with surgical standby, she was orally intubated with a 6.0 mm-endotracheal tube over a fiberoptic laryngoscope.

The patient was transferred to a tertiary care hospital, where the antibiotics were changed to ampicillin-sulbactam, clindamycin, and gentamicin. Computed tomography (CT) scan of the neck and chest showed extensive soft tissue swelling from the oropharynx to the supraglottic region with obliteration of the airway surrounding the endotracheal tube. The adjacent parapharyngeal and cervical soft tissues down to the upper chest were also involved, but no discrete abscess was identified. Bilateral pulmonary infiltrates, atelectasis, and small pleural effusions were present, but the mediastinum was normal.

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The next day, two blood cultures drawn before administration of antibiotics grew *N. meningitidis,* serogroup Y (confirmed by the Massachusetts Department of Public Health Laboratory). The organism was sensitive to penicillin and ceftriaxone and resistant to tetracycline by the Kirby-Bauer method. Sputum culture done by endotracheal tube (after the start of antibiotics) was negative. Close family contacts and the community hospital staff members involved with airway procedures were given prophylactic antibiotics, according to guidelines (rifampin 600 mg PO bid x 4 doses, ciprofloxacin 500 mg PO x 1 dose, or ceftriaxone 250 mg intramuscularly x 1 dose) (7).

Clindamycin and gentamicin were discontinued. Repeat CT scan on hospital day 5 was unchanged, but by day 7, the swelling had resolved, and the patient was extubated in the operating room. She recovered fully and was discharged on hospital day 9; she received i.v. ampicillin-sulbactam at home for an additional 7 days. She remained well, as determined by telephone contact 13 months later. Complement testing (CH-50) 13 months later was normal.

Conclusions

The diagnosis of supraglottitis is most consistent with the patient's clinical picture of sore throat, dysphagia, fever, muffled voice, and swollen supraglottic tissues, as seen on plain films, fiberoptic laryngoscopy, and cervical CT scan (8). However, supraglottitis is uncommonly accompanied by such dramatic external cervical swelling. Ludwig's angina was initially considered, but this diagnosis was unlikely because odontogenic infection and involvement of the sublingual or submandibular spaces were lacking. Cervical abscesses can complicate supraglottitis (9), but no abscess was detected on repeated CT scans. Necrotizing fasciitis was a possibility, especially since the erythema and swelling overlying the chest suggested mediastinal extension. In the one reported case of supraglottitis with cervical necrotizing fasciitis (10), fascial gas was demonstrated on CT scan. Surgical drainage was required for cure; anaerobic organisms were the likely cause. Furthermore, *N. meningitidis,* which rarely causes cellulitis, has not been reported to cause necrotizing fasciitis. The pathologic process in bacterial supraglottitis is cellulitis of the epiglottis and the surrounding upper airway. In this patient, the cellulitis was so aggressive that it extended posteriorly to the spine, anterolaterally to the cervical skin, cephalad to the pharynx, and caudad to the chest.

Cultures of the epiglottis and pharynx were not performed, so the meningococcal bacteremia may have reflected secondary or coincident infection, not supraglottic infection. However, even when laryngeal cultures are performed, organisms isolated from the blood and the throat correlate poorly (11). Although the CT lung scan demonstrated pulmonary infiltrates, bacteremic meningococcal pneumonia complicating supraglottitis due to another organism is improbable.

A search of Medline (the National Library of Medicine's bibliographic database of biomedical journals) from 1966 through mid-1999 located five reports of meningococcal supraglottitis: two from Colorado (2,4), one from Ohio (3), one from Singapore (5), and one from Helsinki, Finland (6). Including our patient in the series of six, the ages of the patients (three were women) were 44, 54, 60, 65, 81, and 95. Two patients had type 2 diabetes mellitus, but the others were otherwise healthy; all had fever, sore throat, and evidence of upper airway compromise. No upper airway cultures were reported, but blood cultures in all six cases were positive for *N. meningitidis*: serogroup B (two), serogroup Y (three), and unreported serogroup (one). No clinical evidence of meningitis or meningococcemia was found in any of the cases. Only our patient had external cervical cellulitis. All patients recovered with appropriate antibiotic treatment. The possibility of complement deficiency was investigated and ruled out in two of two patients. Two patients were treated with steroids i.v. Five of the six patients required airway intervention (three, intubation; two, urgent tracheostomy), a much higher proportion than that in a population-based case series of adults with supraglottitis (11). Thus, bacteremic meningococcal supraglottitis appears to be fulminant and life-threatening.

Most meningococcal disease in the United States is sporadic. One third of all cases occur in adults, who are usually immunocompromised (e.g., by complement deficiency, corticosteroid use, or HIV infection). In one population-based study, more than half the adults had neither rash nor meningitis. Pneumonia, sinusitis, and tracheobronchitis were the main sources of bacteremic meningococcal disease. Supraglottitis

was not observed (12). In particular, the proportion of meningococcal infections due to serogroup Y has been increasing nationally in the last several years. Serogroup Y is frequently associated with meningococcal pneumonia in both civilian (13) and military populations (14).

Why *N. meningitidis* is not a more frequent cause of supraglottitis is not known. The organism is a common colonizer of the upper airways of healthy persons, and the pharynx is the suspected portal of entry of invasive and disseminated disease (1). The organism may also cause simple pharyngitis (15).

The pathogenic determinants of both meningococcal disease and supraglottitis are complex and largely undefined, so we can only speculate how meningococci might cause this syndrome. Meningococcemic syndromes seem to require both epithelial and endothelial invasiveness so that the organism can cross the nasopharyngeal mucosal barrier, enter the bloodstream, and invade other blood vessel walls to produce the characteristic vasculitic organ damage. In contrast, meningococcal isolates from supraglottic syndromes seem to have relatively greater epithelial invasiveness, a propensity for contiguous local inflammatory spread, and decreased tropism for endothelial cells; these characteristics result in a more locally aggressive but less disseminated disease. The presence of various surface-expressed virulence factors (e.g., capsule, pili, cell surface proteins, and lipo-oligosaccharides) that mediate the organism's interaction with certain host cells may explain these differing pathophysiologic properties. For example, certain Opa cell surface proteins facilitate invasion of epithelial cells, while Opc proteins are more efficient at promoting invasion of endothelial cells (16).

N. meningitidis can cause an inflammatory conjunctivitis that progresses to septicemia in approximately 10% of cases (17). In an analogous manner, it can (rarely) extend locally to produce a periorbital cellulitis. In one such case, isolates from the blood and periorbital aspirate of the same patient were identical except for their expression of Opa proteins and their lipo-oligosaccharide phenotype (18).

Host factors (e.g., specific immune system deficiencies) could also be responsible for different disease manifestations. The most well-known example is the susceptibility of patients with terminal complement component deficiencies to neisserial infections. However, these patients have recurrent, but typical meningococcemia, so this deficiency would not be expected to contribute to the supraglottitis syndrome (19).

While *N. meningitidis* has been known for nearly 2 centuries and blood cultures have been routinely available for decades, meningococcal supraglottitis had not been reported until 1995 (2). In contrast, incidence of supraglottitis due to *Haemophilus influenzae* has remained constant in adults 18 years of age or older (11). Furthermore, while one case was reported each year from 1995 to 1997, three cases have been reported in 1998-1999, from three continents, suggesting the emergence of a new meningococcal syndrome worldwide. Surveillance is needed to determine if meningococcal supraglottitis will become more than just a rarity.

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Genetic Evidence of Dobrava Virus in *Apodemus agrarius* in Hungary

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Using nested polymerase chain reaction, we sequenced Dobrava virus (DOB) from the rodent *Apodemus agrarius* in Hungary. The samples we isolated group with DOB samples previously isolated from *A. flavicollis*. This grouping may indicate host switching.

Hantaviruses, the causative agents of hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome, are serologically related viruses of the family *Bunyaviridae* and have a worldwide distribution. Unlike other bunyaviruses, hantaviruses are not transmitted by arthropod vectors. The virus is excreted in the saliva, urine, and feces of infected rodents. Humans become infected by inhalation of aerosols of dried excreta, inoculation through the conjunctiva, or entry through broken skin (1).

Each viral species within the genus *Hantavirus* is primarily associated with a single rodent species, although accidental infections have been reported in other mammals (2). Four primary reservoirs for hantaviruses are found in Europe: *Rattus norvegicus,* Seoul virus; *Clethrionomys glareolus,* Puumala virus (PUU); *Apodemus flavicollis,* Dobrava virus (DOB); and *Microtus arvalis,* Tula virus (TUL) (3). An additional rodent species, *Apodemus agrarius,* is the primary reservoir of Hantaan virus (HTN), the causative agent of Korean hemorrhagic fever, which is found throughout Korea and China but not in Europe. Recently, DOB was isolated from *A. agrarius* in Estonia (4) and Russia (5).

Hantaviruses are present in Hungary; however, their particular virus strains or species and their distribution are unknown. PUU has been confirmed in western Hungary, but the species in eastern Hungary have not been determined (6).

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During field surveys of indigenous rodents in areas used by NATO forces between 1995 and 1996, nine rodents were collected at Tazar Air Force Base, outside Kaposvar, eastern Hungary. A total of 210 trap nights resulted in a trap return of 4.28%. Two *A. agrarius*, four *Microtus agrestis*, and three *Mus musculus* were captured. Animals were live-trapped and euthanized with halazone. Tissue was collected from lungs and kidneys; urine, if present, was also collected.

Total RNA was extracted from collected tissues by using the FastRNA Kit–Green (Bio 101), according to the supplier's recommendations. RNA was converted to cDNA and amplified by the Titan One Tube RT-PCR System (Boehringer, Mannheim, Germany), according to the manufacturer's recommendations. Degenerate primers M-4 (ATGAARGCNGAWGA RNTNACMCCNGG) and M-9 (TGRYCNAGYTG TATYCCCATWGATTG) were used to amplify a 583-bp section corresponding to nt positions 605 to 1,188 in the S segment of HTN strain X95077. No DOB strains were used in the laboratory.

A target sequence of 397 bp (nt from 692 to 1,089) was amplified by nested polymerase chain reaction, and both strands of the amplicons were sequenced. Amplified bands of the predicted size were demonstrable in all tissue samples investigated from the two specimens of *A. agrarius* (designated Tazar-2, Tazar-8). All other samples tested negative. Sequence validity was confirmed by sequencing amplified lung and kidney products from each infected animal. The respective sequences from Tazar-2 and Tazar-8 differed in 3 of the 321 nucleotides examined

(99% identity). Both sequences were aligned with the corresponding S-segment section of several DOBs and five other European and Asian hantaviruses.

The nucleotide sequence identities between Tazar virus (found in this study) and related viral lineages (Figure) included Russian DOB from *A. agrarius,* 88%; Estonian DOB from *A. agrarius,* 86%-87%; Bosnia DOB from *A. flavicollis,* 88%; Greek DOB from *A. flavicollis,* 88%-85%; Sapporo rat virus, 70%; HTN, 68%; Khabarousk, 57%; PUU, 56%; TUL, 53%; and Sin Nombre, 48%. These results indicate that, on the basis of sequence similarity, both Tazar samples are hantaviruses most closely related to DOB. Although the sequence data are limited, phylogenetic analyses linked Tazar-2 and Tazar-8 isolated from *A. agrarius* to a group of DOB previously isolated from *A. flavicollis*. The *A. agrarius* DOB from Russia (5) did not support monophyly (common ancestry) for DOB isolated from *A. agrarius* populations in Hungary and Russia. Other representative hantaviruses, including PUU, TUL, HTN, and Sapporo rat virus, were more basal in the phylogeny (Figure).

HTN infects *A. agrarius* populations in Asia but has not been isolated in Europe. Direct enzyme-linked immunosorbent assay has demonstrated the presence of Hantaan-like antigens in *A. agrarius* in the former republic of Czechoslovakia (5.5%, [7]), the European regions of the former Soviet Union (5.3%, [8]) (28.5%, [9]), and Serbia (2.2%, [10]). Because HTN sequences have not been reported in Europe and HTN and DOB have similar immunologic responses, the earlier findings of Hantaan-like antigens in Europe may represent a more widespread occurrence of DOB in European populations of *A. agrarius*. Recent verification of DOB in populations of *A. agrarius* in Estonia (4), Russia (5), and now Hungary supports this conclusion.

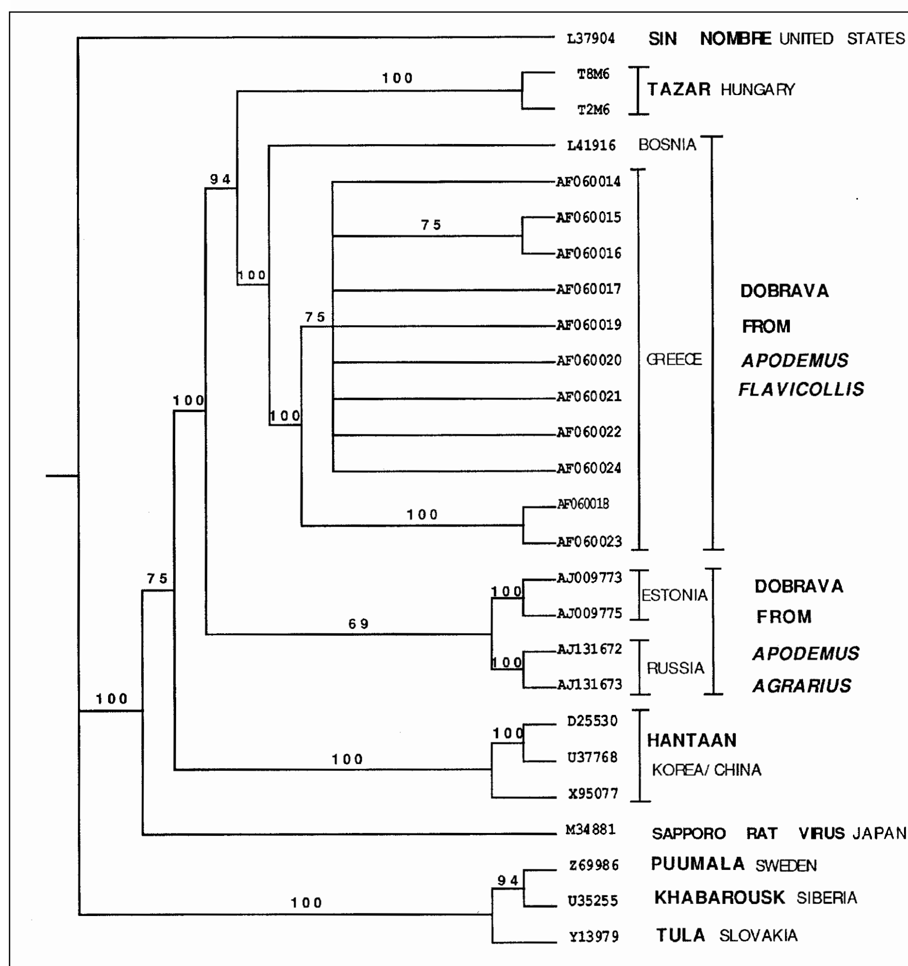

Figure. Cladogram derived from nucleotide sequences of Tazar-2, Tazar–8, and other hantaviruses. Numbers denote Genbank accession numbers. The cladogram was derived from the neighbor-joining estimated phylogeny and bootstrap analysis using p-distance estimates. The phylogenetic analysis was performed by using PAUP 3.1.1 (vers. 4.0.0d64). Numbers at each internode or bifurcation represent bootstrap support based on 1,000 replicates. A Sin Nombre virus sequence (L37904) was used as the outgroup to root the tree. Tazar 2 and Tazar 8 have been submitted to Genbank and received accession numbers AF085336 and AF085337, respectively.

The occurrence of DOB in both *A. agrarius* and *A. flavicollis* provides an opportunity to evaluate the hypothesis concerning distribution of hantaviruses in related rodent hosts. Phylogenetically different Sin Nombre–like viruses have been found in different populations within species of peromyscine rodents that vary ecologically and geographically throughout their range (11). Although these authors found evidence of cospeciation between the rodent host phylogeny and the host-borne hantavirus phylogeny, evidence of host switching was observed with *Peromyscus leucopus*–borne New York virus grouped with *P. maniculatus*–borne viruses rather than with other *P. leucopus*–borne viruses.

Our phylogenetic analysis (Figure) indicates a closer relationship between the *A. agrarius* DOB from Hungary and *A. flavicollis* DOB, with Russian/Estonian DOB representing a sister-group to this clade. This lack of monophyly for the *A. agrarius* DOB may suggest host switching between *A. agrarius* and *A. flavicollis* similar to that between *P. leucopus* and *P. maniculatus*. Nevertheless, the relationships between the isolated DOB lineages suggest a more basal position for the *A. agrarius* DOB than for *A. flavicollis* DOB lineages.

A. flavicollis ranges throughout much of Western Europe eastward to the Ural Mountains, and *A. agrarius* ranges from Eastern Europe eastward to the Pacific Ocean, covering most of the Asian continent (12). Given the extensive range of both species, an examination of other populations within each species, as well as other species of *Apodemus*, might allow correlation of the viral and rodent host phylogeny. The pattern of divergence for hantaviruses in New World peromyscine rodent species may be mirrored in Old World arvicoline rodents. Therefore, the existence of more than one hantavirus in *A. agrarius* may reflect geographic variation within the species or host switching in regions where two host species are potentially sympatric.

Dr. Scharinghausen, an active-duty Army captain, is completing his Ph.D. at Texas A&M University in Wildlife and Fisheries Sciences. His area of expertise is mammalogy, and his research interests include zoonoses and molecular biology.

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Bacterial Resistance to Ciprofloxacin in Greece: Results from the National Electronic Surveillance System

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According to 1997 susceptibility data from the National Electronic System for the Surveillance of Antimicrobial Resistance, Greece has high rates of ciprofloxacin resistance. For most species, the frequency of ciprofloxacin-resistant isolates (from highest to lowest, by patient setting) was as follows: intensive care unit > surgical > medical > outpatient. Most ciprofloxacin-resistant strains were multidrug resistant.

Soon after the broad-spectrum, highly effective antibiotics fluoroquinolones were introduced, their extensive use and misuse in hospitals and communities, as well as in veterinary medicine, have led to the emergence and spread of resistant strains (1,2). Highly divergent rates of fluoroquinolone resistance in both community-acquired and nosocomial pathogens have been reported worldwide (2). Many factors, including patient characteristics, local epidemiologic factors, antibiotic policies, over-the-counter use (which often leads to inadequate use), lower standard of living in developing countries, lack of information on the prudent use of antibiotics, and use of antibiotics in animal husbandry may contribute to the emergence of quinolone-resistant organisms.

Surveillance is an integral part of controlling resistance, and local and national surveys to identify, monitor, and study the epidemiology of the emergence and spread of resistant isolates are needed (3). To identify national trends and local differences in the epidemiology of quinolone resistance in Greece, we report 1997 ciprofloxacin susceptibility data from the National Electronic System for the Surveillance of Antimicrobial Resistance.

The National Electronic System for the Surveillance of Antimicrobial Resistance was introduced in Greece 3 years ago. Involving 17 hospitals throughout Greece, the system analyzes the routine results of the antibiotic sensitivity tests performed in hospital microbiology laboratories by using WHONET software (4).

In our analysis we included 11,097 isolates (4,204 from medical wards, 2,897 from surgical wards, 1,724 from intensive care units [ICU], and 2,272 from outpatient departments) (Table 1). We focused on the bacteria most frequently encountered in Greek hospitals (National Electronic System for the Surveillance of Antimicrobial Resistance [www.mednet.gr/whonet]; N.J. Legakis, Enare Sentry, unpub. data): *Escherichia coli, Klebsiella pneumoniae, Enterobacter* species, *Pseudomonas aeruginosa,*

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Table 1. Isolates included in the analysis[a]

Species	Type of ward				
	Medical	Surgical	ICU[b]	Outpatients	All
Escherichia coli	2,100	1,114	94	1,571	4,879
Pseudomonas aeruginosa	672	527	570	195	1,964
Staphylococcus aureus	452	467	318	248	1,485
Enterobacter spp.	396	332	198	142	1,068
Klebsiella pneumoniae	419	224	177	96	916
Acinetobacter spp.	165	233	367	20	785
All	4,204	2,897	1,724	2,272	11,097

[a]One isolate per species per patient (the first isolated) is shown. [b]ICU, intensive care unit.

[1]G. Antoniadis, E. Arhondidou, S. Chatzipanagiotou, E. Chinou, A. Chrysaki, V. Daniilidis, G. Genimata, H. Gessouli, P. Golemati, E. Kaili-Papadopoulou, A. Kansuzidou, D. Kailis, E. Kaitsa, M. Kanelopoulou, Sp. Kitsou-Kyriakopoulou, Z. Komninou, E. Kouskouni, Chr. Koutsia-Karouzou, S. Ktenidou-Kartali, V. Liakou, H. Malamou-Lada, H. Mercuri, C. Nicolopoulou, A. Pagkali, E. Panagiotou, E. Papafragas, A. Perogamvros, C. Poulopoulou, D. Sofianou, G. Theodoropoulou-Rodiou, S. Thermogianni, E. Trikka-Graphakos, O. Vavatsi-Manou, M. Ventouri, E. Vogiatzakis, A. Xanthaki, Chr. Zagora, E. Chatzidaki, G. Papoutsakis.

Acinetobacter baumanii, and *Staphylococcus aureus*. These species are also the most important nosocomial pathogens in most parts of the world in terms of rate of isolation, pathogenicity, and virulence (5,6).

Isolation and identification were performed by standard methods at the microbiology laboratories of each hospital participating in the network. The susceptibility testing methods were Kirby-Bauer disk diffusion (7 hospitals); Sensititre (Sensititre, Salem, NH) (1); Pasco (Difco, Detroit, MI) (8); and VITEK (Bieux-Merieux Marcy l'Etoile, France) (1). The actual zone diameters or MICs (not the interpretations of the tests) were entered into WHONET. The chi-square test was used to evaluate differences in resistance rates between types of wards, as well as between clinical specimens. Pearson's correlation coefficients were calculated for possible associations between resistance rates and hospital size.

The resistance rate to ciprofloxacin by type of ward, clinical specimen, and bacterial species is shown in Table 2. There is a stepwise decrease in the frequency of isolation of ciprofloxacin-resistant isolates (ciprofloxacin resistance in

Table 2. Ciprofloxacin resistance by specimen and type of ward[a]

	Outpatients		Medical		Surgical		ICU	
	No.	%R[b]	No.	%R	No.	%R	No.	%R
Escherichia coli								
Urine	1,191	5.0	1,572	5.5	597	8.5	39	10.2
Blood	-		195	6.9	14	18.1	5	0.0
Respiratory	-		56	2.1	-		23	9.0
Pus	-		33	12.1	203	8.4	11	27.8
Other	380	4.5	244	7.5	300	6.5	16	20.0
All	1,571	3.7	2,100	5.6	1,114	8.2	94	13.3
Salmonella spp.								
Stool	195	0.7						
Klebsiella pneumoniae								
Urine	62	6.6	254	15.5	85	19.8	28	64.0
Blood	-		45	11.3	10	9.8	18	72.3
Respiratory	-		62	9.8	12	50.0	90	69.8
Pus	-		14	50.0	42	19.0	0	0.0
Other	34	3.1	44	18.5	79	28.3	41	65.4
All	96	5.4	419	15.8	226	23.9	177	67.7
Serratia marcences								
All			76[c]	7.7[c]			20	45.2
Enterobacter spp.								
Urine	76	12.0	190	29.7	85	32.0	24	75.4
Blood	-		37	21.8	13	54.2	24	66.6
Respiratory	-		76	6.3	10	40.2	58	48.6
Pus	-		22	36.8	138	18.5	27	67.6
Other	66	10.8	71	16.9	86	23.3	65	69.0
All	142	11.6	396	22.2	332	24.8	198	62.2
Pseudomonas aeruginosa								
Urine	51	31.0	270	44.0	171	40.7	70	79.3
Blood	0	0.0	24	20.6	13	46.5	29	75.6
Respiratory	11	18.2	258	34.4	29	44.6	379	62.9
Pus	18	11.3	35	31.6	147	22.6	16	69.5
Ear	72	1.7	7	47.3	30	3.7	0	0.0
Other	43	18.8	78	26.9	137	25.9	76	66.9
All	195	16.7	672	37.5	527	28.2	570	66.4
Acinetobacter spp.								
Urine	-		72	62.6	32	65.9	34	94.4
Blood	-		18	38.7	16	69.0	40	92.3
Respiratory	-		38	49.7	11	100.0	190	91.0
Pus	-		13	61.8	87	60.1	19	94.8
Other	-		24	62.5	87	69.1	84	78.9
All	20	45.1	165	56.8	233	66.6	367	88.4
Staphylococcus aureus								
Urine	-		37	32.9	16	31.0	-	
Blood	-		101	51.0	15	67.0	40	62.7
Respiratory	-		123	45.3	28	57.1	221	65.8
Pus	104	18.2	88	21.6	272	30.8	14	71.4
Ear	52	3.8	-		-		-	
Other	92	10.3	103	25.6	136	31.4	43	67.4
All	248	12.8	452	30.5	467	33.0	318	63.6
MRSA[d]	40	56.7	140	69.1	176	75.3	375	94.3
MSSA[e]	184	1.7	256	12.4	219	6.5	92	4.6

[a]One isolate per patient (the first isolated) is shown. [b]R, resistant. [c]Medical and surgical wards combined. [d]MRSA, methicillin-resistant *S. aureus*. [e]MSSA, methicillin-sensitive *S. aureus*.

isolates from ICU patients > isolates from surgical patients > isolates from medical patients > isolates from outpatients). These differences were significant ($p < 0.01$), with the exception of decreases in resistance rates for *E. coli* between surgical wards and ICUs; for *Enterobacter* spp. between medical and surgical wards; for *Acinetobacter* spp. between outpatients, medical, and surgical wards; and for *S. aureus* between medical and surgical wards. Moreover, for *P. aeruginosa,* the resistance rates were significantly higher in medical than in surgical wards ($p = 0.00097$).

As for clinical specimens, each bacterial species followed a different pattern (Table 2). In medical wards, enterobacterial strains isolated from purulent infections were more often resistant to ciprofloxacin, but this difference was statistically significant only for *K. pneumoniae* ($p = 0.012$). In surgical wards, blood and respiratory isolates were more often resistant, but this difference was significant only for *Enterobacter* spp. ($p = 0.02$). On the other hand, ciprofloxacin-resistant *P. aeruginosa* strains were more frequently isolated ($p = 0.0021$) in medical wards from urine and in surgical wards from urine and blood as opposed to all other specimens ($p = 0.0005$). No significant differences were observed in the rate of isolation of ciprofloxacin-resistant *A. baumanii* strains among the various clinical specimens. *S. aureus* strains resistant to ciprofloxacin were mostly methicillin-resistant (MRSA) (Table 2). Very low resistance rates were observed in *P. aeruginosa* isolated from ear infections, especially from outpatients.

Approximately 75% of *K. pneumoniae*, 87% of *Enterobacter* spp., 55% of *P. aeruginosa*, 76% of *A. baumanii*, and 75% of MRSA strains were drug resistant to at least three different classes (Table 3). However, 15% of the ciprofloxacin-resistant *E. coli* were resistant only to this antibiotic, and 25% had additional resistance only to cotrimoxazole. Moreover, 48% of ciprofloxacin-resistant but methicillin-sensitive *S. aureus* were resistant only to chloramphenicol.

When we plotted resistance rates to ciprofloxacin against the number of beds in each hospital, we found no correlation (Figure). The rate of isolation of ciprofloxacin-resistant isolates varied greatly by hospital for all species examined: from 1% to 15% for *E. coli*, 1% to 23% for *K. pneumoniae*, 1% to 33% for *Enterobacter* spp., 11% to 33% for *P. aeruginosa*, 29% to 73% for

Table 3. Resistant phenotypes of ciprofloxacin-resistant isolates to other classes of antibiotics[a]

Klebsiella pneumoniae

Phenotype[b]	No.	%
F	4	3.7
DBXF	9	8.4
IDB F	16	15.0
IDBXF	64	59.8
all other	14	13.1
All	107	100.0

Enterobacter spp

Phenotype	No.	%
F	0	0
IF	4	2.5
IDB F	7	4.4
IDBXF	131	82.9
all other	16	10.1
All	158	100.0

Escherichia coli

Phenotype	No.	%
F	25	15.1
IDBXF	16	9.6
IXF	29	17.5
XF	42	25.3
all other	54	32.5
All	166	100.0

Pseudomonas aeruginosa

Phenotype	No.	%
F	10	7.3
1DM F	14	10.2
1DMNF	23	16.8
1 M F	40	29.2
all other	50	36.5
All	137	100.0

Acinetobacter baumanii

Phenotype	No.	%
F	0	0.0
SMD XF	5	10.0
D XF	15	30.0
MD XF	23	46.0
all other	7	14.0
All	50	100.0

Staphylococcus aureus

MRSA			MSSA		
Phenotype	No.	%	Phenotype	No.	%
F	0	0.0	F	7	10.3
OG E F	23	11.3	E F	9	13.2
OG ECF	44	21.7	CF	33	48.5
OGXECF	84	41.4			
all other	52	25.6	all other	19	27.9
All	203	100.0	All	68	100.0

[a]All wards, intensive care units isolates are not included. [b]1, piperacillin; B, tobramycin; C, chloramphenicol; D, ceftazidime; E, erythromycin; F, ciprofloxacin; G, gentamicin; I, cefoxitin; M, amikacin; N, imipenem; O, oxacillin; S, amoxicillin/sulbactam; X, cotrimoxazole; MRSA, methicillin-resistant *S. aureus;* MSSA, methicillin-sensitive *S. aureus*.

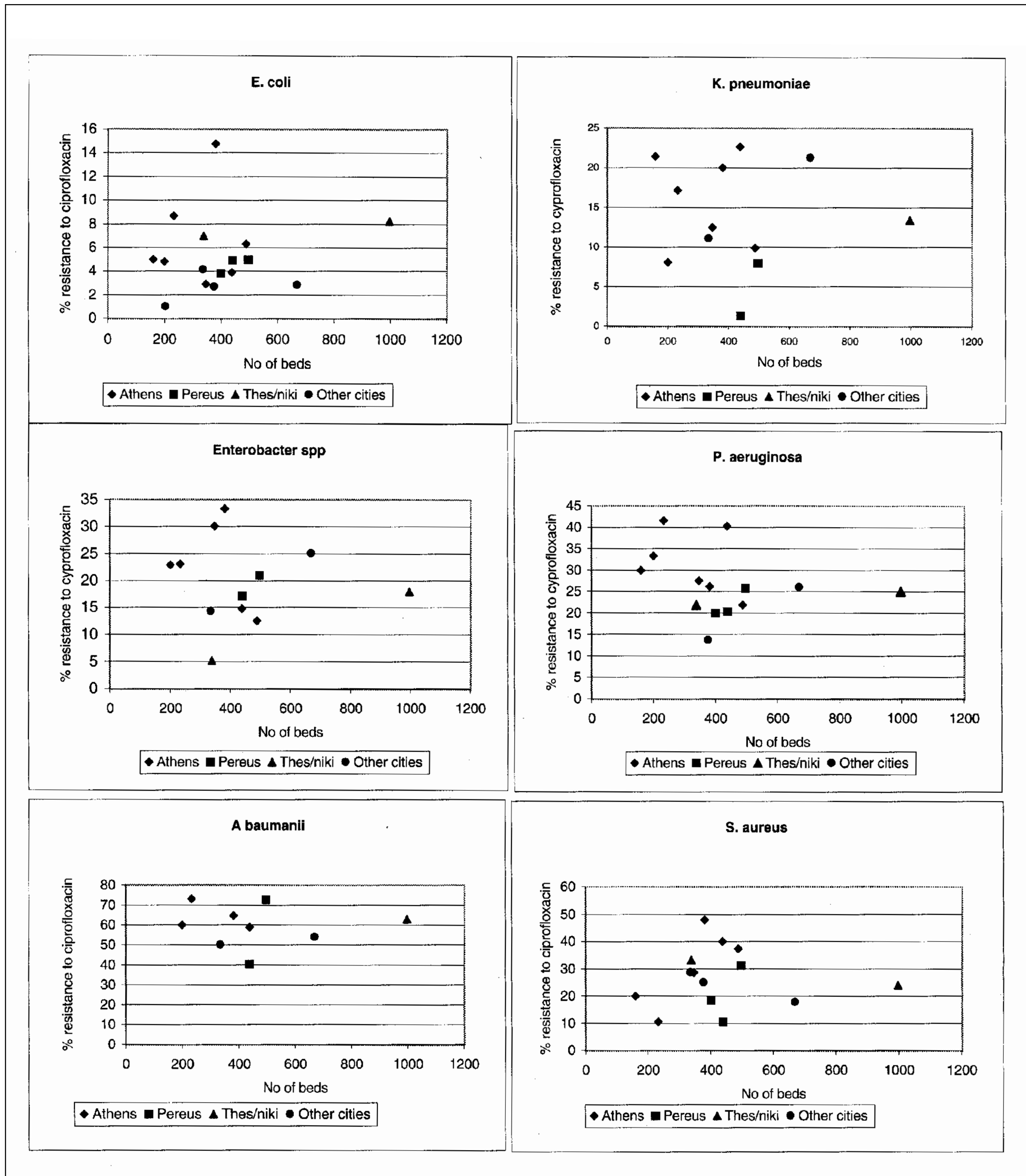

Figure. Resistance rates to ciprofloxavin in each hospital by number of beds and geographic area of the hospital. Only hospitals with more than 20 isolates are included. (Isolates from all wards but not intensive care units.)

A. baumanii, and 11% to 48% for *S. aureus*. Ciprofloxacin resistance was observed in hospitals throughout Greece.

In Europe and North America, a striking difference in the incidence of bacterial resistance to quinolones has been observed between nosocomial and community-acquired infections; resistance is only rarely encountered among the latter (2,7). The incidence of resistance to fluoroquinolones in bacteria isolated from hospital-acquired infections varies among bacterial species, clinical settings, and countries and may be related to local epidemic spread of a few clones (2). The highest incidence of resistance is among *P. aeruginosa, Acinetobacter* spp., *Serratia marcescens,* and particularly MRSA strains (8). Our results place Greece among the countries with high resistance levels to quinolones. Although quinolones are among the antibiotics restricted by the Greek Ministry of Health and Welfare, the mean national level of quinolone resistance has increased in most bacterial species during the last 5 years (9).

The 3.7% quinolone resistance rate among *E. coli* isolated from outpatients is almost double that in other industrialized countries (2). This high rate may be due to the use of quinolones, and especially norfloxacin, as a first-line antibiotic in Greece to treat uncomplicated urinary tract infections in the outpatient setting. Free access to fluoroquinolones has also been incriminated in increased quinolone resistance in industrialized and developing countries (10,11). The low rate of quinolone resistance in salmonellas, compared with other countries (12,13), may be due to infrequent use of quinolones in farm animals in Greece. Among *Enterobacteriaceae,* quinolone resistance seems to be higher in *K. pneumoniae* and *Enterobacter* spp. than in *S. marcescens*.

The high level of resistance in ICUs was expected since ICUs are well-known focuses of antimicrobial resistance (14). Hospitalization in ICUs was an independent risk factor for acquiring infection by multidrug-resistant strains in Greece (15). Moreover, ICU patients are often colonized with endemic, multidrug-resistant strains, which often spread to other wards (16).

We found higher rates of isolation of quinolone-resistant strains of some species in the surgical wards than in medical wards. Patients at high risk for a resistant nosocomial infection (e.g., cancer patients, immunosupressed patients) are usually in medical wards. High resistance in the surgical wards could be the result of nursing practices or unnecessary prophylactic administration of antibiotics, both of which should be further evaluated.

Most quinolone-resistant strains in Greece are also resistant to other clinically relevant antibiotics. The possible clinical and epidemiologic importance of the newly described multidrug efflux pumps in multidrug resistance, mainly in *P. aeruginosa,* is under investigation worldwide (17). Moreover, the marginal susceptibility of *S. aureus* to quinolones and the ease with which mutations affecting susceptibility can occur in this species contribute to the observed high rates of quinolone resistance. MRSA strains are no more likely to develop resistance to quinolones than other staphylococci (8). In any case, the favorable accumulation of different traits in quinolone-resistant strains or, alternatively, the favorable potential for mutation to quinolone resistance in multidrug-resistant strains has not been proved. Epidemiologic parameters, and more specifically the sequential introduction of various antibiotic classes in most of the world and in Greek hospitals, could explain multidrug resistance. The extensive aminoglycoside and beta-lactamase use in the 1980s is responsible for the high prevalence of multidrug-resistant plasmids and transposons found in the nosocomial strains of various bacterial genera in Greek hospitals (18-20). The strains harboring these plasmids can survive in the hospital environment and become the best candidates for selection of resistant mutants under the pressure of quinolones.

That quinolone-resistant strains are found in hospitals in all parts of Greece and resistance is not associated with the size of the hospital or its geographic area are consistent with the high prescription rate for quinolones. However, the isolation rate of resistant strains varied considerably by hospital, perhaps because of local epidemiologic factors (e.g., prescribing or nursing habits) or possible (epidemic) spread of strains among patients.

This study has limitations. First, it is based on routine data generated in the microbiology laboratories of participating hospitals. Sometimes different antibiotics are tested in each hospital, which limits the possibility for interhospital comparisons. Moreover, different methods for susceptibility testing are used in

each hospital. Data such as antibiotic consumption or days of hospitalization are not available since they are not included as information in the WHONET software and they are difficult and time-consuming to collect routinely.

Quinolone use is a well-proven independent risk factor for resistance (21,22). Nevertheless, local differences indicate that other epidemiologic parameters should be further evaluated.

The National Electronic System for the Surveillance of Antimicrobial Resistance has been supported in part by a grant from the Greek Ministry of Health and Welfare.

The following hospitals participate in the system: Polycliniki General Hospital, Agia Olga General Hospital, Elpis General Hospital, First IKA Hospital of Athens, Agios Savas Cancer Hospital, Sismanoglion General Hospital, Hippocration General Hospital, Areteion University Hospital, Venizelio General Hospital, University Hospital of Alexandroupolis, University Hospital of Ioannina, General Hospital of Xanthi, Threassio General Hospital, Tzannio General Hospital, Asclepeion Voulas General Hospital, Theagenio Cancer Hospital, and Hippocration Hospital Thessaloniki.

Dr. Vatopoulos is a medical microbiologist and assistant professor in the Department of Hygiene and Epidemiology, Medical School, Athens University. His chief research interest is the molecular epidemiology of antibiotic resistance in bacteria (mainly gram-negative). He is now involved in the establishment and operation of an electronic network for the surveillance of antibiotic resistance in Greece.

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Emergence of Related Nontoxigenic *Corynebacterium diphtheriae* Biotype *mitis* Strains in Western Europe

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We report on 17 isolates of *Corynebacterium diphtheriae* biotype *mitis* with related ribotypes from Switzerland, Germany, and France. Isolates came from skin and subcutaneous infections of injecting drug users, homeless persons, prisoners, and elderly orthopedic patients with joint prostheses or primary joint infections. Such isolates had only been observed in Switzerland.

Nontoxigenic *Corynebacterium diphtheriae* strains were recovered from approximately 1 per 1,000 throat swabs from immunized British military personnel in Germany from 1993 to 1995 (1). Such nontoxigenic *C. diphtheriae* biotype *mitis* isolates had been described in skin, throat, and blood cultures of Swiss injecting drug users; 32 of the isolates belonged to the same clone (2,3). Our study demonstrates that this clone and closely related clones occurred between 1990 and 1997 in two other European countries, and only sometimes in persons with poor hygiene.

Five *C. diphtheriae* isolates came from two laboratories in Zurich and Bern, Switzerland; 11 from four laboratories in Hamburg, Germany; and 1 from Paris, France (Table). They were identified in Zurich as *C. diphtheriae* biotype *mitis* (4); that is, they were nonlipophilic, were nitrate reductase-positive, and did not ferment glycogen. By polymerase chain reaction techniques (5), the diphtheria toxin gene was not detected in any isolate. For some isolates, the Elek test was also performed; it was consistently negative. For ribotyping, DNA was isolated, digested with either *Eco*RI or *Pvu*II, electrophoresed, blotted, and probed for rDNA as described elsewhere (2,3). Disk susceptibility testing to tetracycline and MIC determinations were done according to National Committee for Clinical Laboratory Standards methods (6). All other bacteria isolated were also identified and serotyped in Zurich.

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Many (10 [59%] of 17) *C. diphtheriae* isolates were from skin or subcutaneous infections (wounds, ulcers) in Swiss patients and were found with *Staphylococcus aureus, Streptococcus pyogenes,* group C/G streptococci, or (sometimes) with gram-negative rods. Most patients in this subgroup were injecting drug users, homeless persons, prisoners, and (with one exception) men with a mean age of 40 (30 to 58) years. Another subgroup (from Germany) consisted of six patients with joint or bone infections (mentioned briefly in an earlier publication on coryneform bacteria from such infections; a seventh patient had *C. diphtheriae* biotype *gravis* [7]). The six had been hospitalized at the Endo Clinic in Hamburg, which specializes in orthopedic surgery. Four had had implantations of hip endoprostheses and had been admitted for prosthetic infections (with coagulase-negative staphylococci or *S. aureus)*; one pure culture of *C. diphtheriae* biotype *mitis* was obtained from a patient with a knee prosthesis, and one mixed culture with *Peptostreptococcus magnus* and *Peptostreptococcus prevotii* was obtained from a patient who had a purulent knee infection after a fracture. Their average age was 60 (23 to 82) years and, to our knowledge, none was a drug user. While the Hamburg isolates were recovered only once from every patient,

Table. Nontoxigenic *Corynebacterium diphtheriae* isolates

Isolate no.	Date isolated[a]	Place of isolation[b]	Ribotyping pattern[c] *Eco*RI	Ribotyping pattern[c] *Pvu*II	Patient's sex, age (yr)	Clinical diagnosis/ source	Other bacteria isolated	Underlying conditions
2012	1990	Paris	A	C	m, 6	Endocarditis/blood culture	----	Skin lesions, scabies
2410	2/1995	Hamburg	A	B	m, 30	Ulcus/lower leg	*Staphylococcus aureus, Streptococcus pyogenes*	Prisoner
2689	3/1995	Hamburg	A	A	f, 82	Puncture/hip joint	*S. epidermidis, Corynebacterium pseudodiphtheriticum*	Total hip joint endoprosthesis
1682	9/1995	Hamburg	A	B	m, 45	Wound swab	*S. aureus, S. pyogenes*	Homelessness
1935	3/1996	Hamburg	A	B	m, 58	Wound swab	*S. aureus, S. pyogenes*	Alcoholism, homelessness
1836	3/1996	Hamburg	A	B	m, 45	Abscess/lower leg	*S. aureus, S. pyogenes*	Injecting drug use
2661	3/1996	Hamburg	A	A	m, 35	Arthroscopy aspirate/knee joint	*Peptostreptococcus prevotii, P. magnus*	Osteosynthesis, arthrotomy, knee joint after trauma
2658	4/1996	Hamburg	A	A	f, 76	Fistula/hip joint	*S. aureus*	Total hip joint endoprosthesis
2674	5/1996	Hamburg	A	A	f, 62	Aspirate/hip joint	*Streptococcus* group C	Total hip joint endoprosthesis
2670	7/1996	Hamburg	A	A	f, 81	Aspirate/knee joint	----	Total knee joint endoprosthesis
2667	7/1996	Hamburg	A	A	m, 23	Swab/hip joint	Coagulase-negative staphylococci	Total hip joint endoprosthesis
2413	11/1996	Hamburg	A	B	m, 36	Ulcus/lower leg	*S. aureus, Streptococcus* group C/G	Prisoner
2446	12/1996	Zurich	A	A	f, 38	Wound/upper leg	*S. aureus, S. pyogenes*	Injecting drug use
2464	1/1997	Zurich	A	A	m, 39	Ulcera/lower arm	*Escherichia coli, S. aureus, S. pyogenes*	Injecting drug use
2473	1/1997	Zurich	A	A	m, 38	Ulcera/leg	*E. coli, Streptococcus* group C, G	Injecting drug use
2475	1/1997	Zurich	A	A	m, 31	Ulcus/pretibial	*Pseudomonas aeruginosa, E. coli, Streptococcus* group C, G	Injecting drug use
480	5/1997	Bern	A	A	m, 39	Ulcus/upper leg	*S. aureus, Clostridium perfringens,* mixed anaerobic flora	Injecting drug use

[a]Month/Year
[b]Paris, France; Hamburg, Germany; Zurich, Switzerland; Bern, Switzerland.
[c]DNA was isolated, digested with either *Eco*RI or *Pvu*II, electrophoresed, blotted, and probed for DNA. Three distinct ribopatterns emerged, each with eight bands: patterns A, B, and C.

their isolation dates stretched from March 1, 1995, until July 26, 1996. Three were isolated on the day of admission, and all of them were isolated by different technologists. Finally, one child (from France) had endocarditis with *C. diphtheriae* biotype *mitis*.

All isolates had identical antimicrobial susceptibility patterns. They were susceptible to amoxicillin (MIC, 0.25 µg/ml), amoxicillin-clavulanic acid (0.06 µg/ml), chloramphenicol (2 µg/ml), ciprofloxacin (0.25 µg/ml), clarithromycin (0.03 µg/ml), clindamycin (0.25 µg/ml), imipenem (0.03 µg/ml), penicillin (0.25 µg/ml), and vancomycin (1 µg/ml). In contrast, all strains were resistant to tetracycline in the disk diffusion test (inhibition zone diameter 10 mm to 11 mm); their MICs for tetracycline, doxycycline, and minocycline were 64 µg/ml, 16 µg/ml, and 16 µg/ml, respectively. While this type of isolated tetracycline resistance was typical for the isolates from Swiss injecting drug users (3), tetracycline resistance in nontoxigenic *C. diphtheriae* has otherwise very rarely been observed in Europe (8). It has, however, been reported in toxigenic *C. diphtheriae* isolates from Indonesia and, rarely, from Canada (9). The

mechanism conferring this resistance in our strains has not been investigated.

On analysis with restriction enzyme *Pvu* II, three different ribopatterns with eight bands each were found among the strains (Figure). These patterns, though distinct, shared five (A vs. C, B vs. C) and six (B vs. A) bands, respectively, and, therefore, may be considered related, as they would be if criteria for pulsed-field gel electrophoresis were applied (10). This view is supported by the fact that all strains had identical *Eco*RI patterns (not shown). All Swiss isolates were identical with the use of both enzymes, whereas both the Swiss pattern and a second pattern were found among the Hamburg isolates. The third pattern was found exclusively in the single French strain. The staphylococcal and streptococcal strains were not typed; they are no longer available to us.

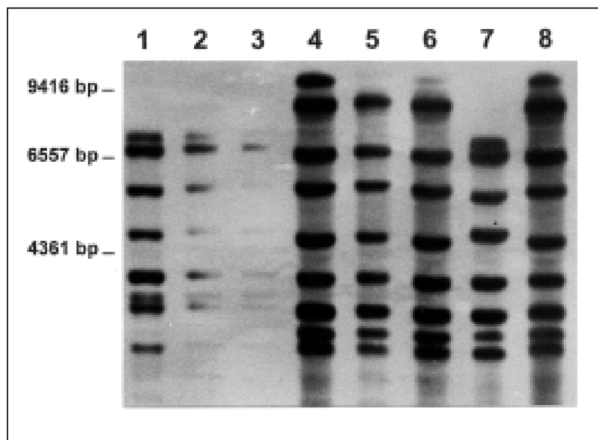

Figure. Ribosomal RNA gene restriction patterns obtained by using restriction enzyme Pvu II of *Corynebacterium diphtheriae* isolates belonging to ribotypes B (lanes 1 to 3), A (lanes 4 to 6 and 8), and C (lane 7).

Our isolates thus resemble those reported from Switzerland between 1990 and early 1996 (2,3), which were also often accompanied by *S. aureus* or beta-hemolytic streptococci. Such nontoxigenic *C. diphtheriae mitis* may cause endocarditis, arthritis, and osteomyelitis (11,12). Most of the 52 isolates from France (11), examined with restriction enzymes different from ours and not available to us, also belonged to one ribotype. Their relatedness to our strains is unknown; however, they were largely tetracycline-susceptible. The two throat isolates from St. Petersburg, Russia, associated with a fatal diphtherialike disease (12) were not typed or tested for antibiotic susceptibility.

The origin of the isolates we describe is unknown. They may have been present (but unrecognized) in the population for a long time. The mode of transmission is most likely common use of drug paraphernalia in the injecting drug use cases and in the endocarditis case; transmission is very difficult to explain in the orthopedic infection subgroup. Although the Swiss and the Danish borders are not close, migration and contacts are not uncommon among injecting drug users. The isolates may also have been distributed through the drugs themselves, as recently reported for *S. pyogenes* in Switzerland (13).

Our study may be representative for Switzerland and Germany, but since submitting *C. diphtheriae* strains to a central reference laboratory in these countries is not mandatory, we cannot estimate the frequency of these strains. These strains may also have spread to other European countries; this hypothesis can only be tested in a large multicenter study of European diphtheria reference laboratories.

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First Case of Human Ehrlichiosis in Mexico

To the Editor: Ehrlichiosis is a zoonotic disease transmitted to humans through the bite of infected ticks (1). The first recognized human ehrlichial infection, Sennetsu fever, was described in Japan in 1954 (2). The first case of human ehrlichiosis in the United States was recognized in 1986 and was reported in 1987 (3). The disease is caused by intracellular gram-negative bacteria of the *Ehrlichia* genus. The bacteria can be found in the monocytes and granulocytes of peripheral blood. Human monocytic ehrlichiosis is caused by *E. chafeensis,* and human granulocytic ehrlichiosis is caused by *E. equi* or *E. phagocytophilia,* which was first recognized in 1994 (4). Most cases occur between April and September, and the reservoirs are field animals such as rodents, deer, and dogs. The clinical spectrum of the disease is similar to that of other febrile illnesses; without adequate and timely treatment, approximately 5% of the patients die (5).

In the United States, more than 400 cases of serologically confirmed *E. chaffensis* infection have been documented since 1996 (6). No cases have been reported in Mexico.

In February 1997, we evaluated a 41-year-old male patient from Merida. The patient had been exposed to ticks during activity in a rural area 1 week before the onset of illness. Clinical manifestations included frequent hyperthermia, rash, myalgia, headache, anorexia, fatigue, and cough. Physical examination showed bilateral cervical lymphadenopathy, and a chest radiograph showed an interstitial bilateral infiltrate. Hematic cytometry showed thrombocytopenia of 134 x 10^3/μL and 3200 leukocytes (1440 neutrophils/μL). Hepatic transaminases were elevated, with an aspartate aminotransferase: 92 U/L (normal: 22 U/L), alanine aminotransferase: 48 U/L (normal: 18 U/L), gamma-glutamyltranspeptidase: 278 U/L (normal: 28 U/L); and globulins: 4.8 g/dL with a polyclonal pattern. No antibodies against rickettsia, dengue virus, B-19 parvovirus, or HIV were detected. A serum sample gave a positive reaction by indirect immunofluorescence assay against *E. chaffeensis* at titers of 1:64 on week 2 and 1:128 on week 3. No infected monocytes or granulocytes were observed in peripheral blood. Remission of the clinical manifestations began on week 4 and was completed on week 6.

This case indicates the existence of human ehrlichiosis in Yucatan, Mexico. Reactivity to *E. chaffeensis* suggests human monocytic ehrlichiosis; however, as antibody testing was not performed with *E. phagocytophila* or *E. equi,* the possibility of human granulocytic ehrlichiosis cannot be excluded. In any event, case reports indicate the need for deliberate search for cases. Dengue is endemic in this area of Mexico, and ehrlichiosis should be considered as a differential diagnosis.

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HIV-1 Subtype F in Single and Dual Infections in Puerto Rico: A Potential Sentinel Site for Monitoring Novel Genetic HIV Variants in North America

To the Editor: Although international efforts to systematically collect, characterize, and classify HIV isolates from around the world have increased considerably, data on HIV-1 genetic variations in Puerto Rico are limited. This island (population 3.7 million) has one of the highest

AIDS incidence rates in the United States (53.3 cases per 100,000) (1). To evaluate the potential for a multiple subtype distribution pattern in Puerto Rico, we analyzed genetic variations between HIV-1 strains isolated from peripheral blood mononuclear cells of 63 asymptomatic HIV-infected female commercial sex workers from 12 communities. These participants were part of 290 female commercial sex workers followed in a larger cross-sectional study of risk behavior (2).

HIV-1 subtypes F (n = 4) and B (n = 44) strains were identified in persons infected with a single viral subtype with a molecular screening assay based on restriction fragment length polymorphism (RFLP) analysis and with DNA sequencing of the viral protease gene-prot (3). The remaining 15 specimens were classified by RFLP as potential dual infections. Further cloning and sequencing of prot from three of these specimens confirmed one dual infection involving subtypes F and B viruses and identified two infections caused by genetically distinct quasispecies of subtype B variants.

In further detailed pairwise analysis of HIV-1 prot genes, a small nucleotide divergence of 0.3% (0.0 to 1.1) within subtype F contrasted with a typical value of 6.3% (5.1 to 7.8) for the intrasubtype distance within subtype B prot sequences (4). The 99% similarity between prot subtype F Puerto Rican sequences suggested an epidemiologic link or a recent introduction of subtype F in Puerto Rico. Comparative sequence analysis of the C2-V3 env is useful in establishing the time that elapsed from infection on the basis of an annual nucleotide divergence of 0.5% to 1% in this region (5). Such analysis has been used to study the epidemiologic link between cases (4,6). Thus, we compared env sequences from two of five persons infected with prot subtype F strains. This analysis provided several observations. Env nucleotide divergence of 13.2% did not support a direct epidemiologic link between these strains. Furthermore, the relatively high intrasubtype diversity between env sequences suggested that evolution from a common progenitor would have taken a minimum of approximately 13 years. Phylogenetic analysis classified these two env sequences as subtype B, indicating that at least some of Puerto Rican prot subtype F viruses represent HIV-1 mosaics involving closely related prot F and significantly divergent env B sequences. Overall, discrepancy in both subtype assignment and nucleotide diversities within prot and env regions may indicate that distinct F/B mosaics circulating in Puerto Rico were likely the result of recombination between highly homogeneous subtype F of relatively recent arrival and divergent resident subtype B viruses.

HIV-1 infections with subtype F strains including B/F mosaics have been reported in Brazil (3,7). To evaluate a potential HIV-1 linkage between Brazil and Puerto Rico, a comparative phylogenetic analysis was done on subtype F viral prot sequences from these countries. This analysis documented that HIV-1 subtype F strains in Puerto Rico are distinct from both Brazilian and Romanian viruses. Furthermore, our results show that genetic analysis of prot allows tracking of subtype F viruses of different origin. Recently, by this approach, HIV-1 prot subtype F of Puerto Rican origin and F prot/B env mosaic were identified in HIV-1-infected persons in New York city (8). Observation of HIV-1 subtype F strains in Puerto Rico together with the recent report describing the first cases of such infections in New York indicates the potential for further emergence of subtype F on the North American continent. The presence of a complex distribution pattern of subtype F infections in Puerto Rico has serious implications for the evaluation and development of HIV diagnostics and vaccines.

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The nucleotide HIV-1 sequences obtained in this study were submitted to GenBank; their accession numbers are AF096813-AF096833.

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Paratyphoid Fever in India: An Emerging Problem

To the Editor: Enteric fever is a major public health problem in India, accounting for more than 300,000 cases per year, *Salmonella typhi* is the most common etiologic agent (1), but *Salmonella paratyphi A,* the other causative agent, causes more asymptomatic infections than *S. typhi*. According to earlier reports from India, *S. paratyphi A* was implicated as a causative agent in 3%-17% of enteric fever cases (2). However, a large community-based study in an urban slum of Delhi during October 1995 to October 1996 found that *S. paratyphi A* caused approximately 20%-25% of the cases of enteric fever in this region (3). An outbreak of enteric fever due to a single *S. paratyphi A* strain in an urban residential area was reported in 1996 from New Delhi, where contaminated water was implicated as the probable source (4,5). This outbreak prompted a retrospective analysis of the laboratory records of the All India Institute of Medical Sciences, New Delhi, over a 5-year period (1994-1998) to study the change, if any, in the etiology of enteric fever in North India.

We evaluated all blood culture records from the institute's clinical bacteriology laboratory for April to October (the months with the highest number of enteric fever cases) each year. Records were from patients residing in New Delhi and the surrounding areas of North India. The blood was collected by a phlebotomist in the outpatient department or by a resident doctor in hospital wards. Blood cultures were carried out by standard laboratory technique (6). Five ml of blood was added to 50 ml of brain heart infusion broth (Hi-Media Laboratory, India) under aseptic conditions. Bacterial identification was accomplished by standard microbiologic protocol (6). Susceptibility to antibiotics (amoxycillin, chloramphenicol, cotrimoxazole, gentamicin, ciprofloxacin, and ceftriaxone) was tested by the comparative disk diffusion method (Stokes method) (7). Chi-square for trend was calculated, and the p value was determined.

The total number of blood cultures performed for enteric fever cases (10,109 in 1994, 12,092 in 1995, 17,652 in 1996, 15,997 in 1997, and 17,012 in 1998) did not change significantly over this period. The isolation of *S. typhi* changed little (Chi-square = 2.367; p = 0.123; statistically not significant). However, the proportion of *S. paratyphi A* isolates rose from 6.5% in 1994 to 44.9% in 1998 (Chi-square = 22.20; p <0.001; statistically significant). The proportion of *S. paratyphi A* isolations in enteric fever cases from 1994 to 1998 was 6.5%, 21.2%, 50.5%, 30.7%, and 44.9%, respectively. Even excluding the strains from the 1996 outbreak (4), we found that the proportion of *S. paratyphi A* in enteric fever cases increased compared with *S. typhi* (Chi-square = 30.528; p <0.001). With our catchment area, case definition of enteric fever, and laboratory methods remaining the same during this period, it appears that the etiology of enteric fever in North India is changing significantly.

The age-wise distribution of *S. typhi* and *S. paratyphi A* showed that *S. typhi* was a significant isolate from children < 5 years of age, while this distribution was not observed for *S. parathyphi A,* which involved those > 5 years

of age. Sex was not significantly associated (mean male to female sex ratio was 32.4:18 for *S. typhi* and 15.8:10.6 for *S. paratyphi A*).

S. typhi has become increasingly sensitive to amoxycillin, chloramphenicol, and gentamicin, increasing from 75.1% in 1994 to 96.6% in 1998 for amoxycillin, from 71.9% in 1994 to 91.6% in 1998 for chloramphenicol, and from 96.4% to 100% for gentamicin. *S. paratyphi A* strains have remained uniformly sensitive (100%) to all antibiotics (amoxycillin, chloramphenicol, and gentamicin, as well as ciprofloxacin and ceftriaxone) used in the treatment of enteric fever. In light of reports of multidrug resistance in *S. typhi,* especially to quinolones, continued surveillance and monitoring of antimicrobial sensitivity of *S. paratyphi A* strains are needed.

The increase in proportion of *S. paratyphi* A cases, which may be due to a high degree of clinical suspicion (with mild fever cases investigated for enteric fever), changing host susceptibility, or even change in the virulence of the organism, should be further investigated.

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Hepatitis C Virus RNA Viremia in Central Africa

To the Editor: Epidemiologic serosurveys have demonstrated high prevalence (6%-15%) of hepatitis C virus (HCV) infection in adults in sub-Saharan Africa (1-4). Although possible false-positive HCV serologic test results have been reported in Africa, HCV prevalence rates suggest a high rate of chronic infection among persons with anti-HCV antibodies (5,6). We have focused on HCV RNA infectivity of blood from donors attending the National Blood Center in Bangui, Central African Republic.

We prospectively tested all blood donors between February and April 1998 for serum anti-HCV antibodies by both an HCV third-generation enzyme-linked immunosorbent assay (ELISA) (Abbott HCV EIA 3.0 test, Abbott, Chicago, IL, USA), which was chosen as a reference test for immunoglobulin (Ig) G antibodies to HCV, and by a simple membrane immunoassay system (Ortho HCV Ab Quik Pack, Ortho Diagnostic Systems Inc., Tokyo, Japan) (7). Anti-HCV-positive serum samples were further subjected to qualitative detection of HCV RNA by reverse transcription-polymerase chain reaction (AMPLICOR-HCV, Roche Diagnostic Systems, Inc., Branchburg, NJ, USA) (8). Of 163 serum samples (mean age ± standard deviation, 30±8 years), 155 were from male blood donors, 83 (51%) from first-time donors, and 125 (77%) from donors in the recipient's family. Fifteen (9.2%; 95% confidence interval [CI] 5%-15%) samples contained IgG to HCV by ELISA. Of the ELISA-positive samples, 14 were positive by the Quik Pack assay (sensitivity, 93.0%); of the 148 remaining ELISA-negative samples, 147 were negative by the Quik Pack assay (specificity, 99.3%). The agreement between the results of the two methods was 98.7%. Of the 163 samples, 10 (6.1%; CI 95%: 3%-11%) were positive for HCV antibodies (by ELISA and rapid test) and for HCV RNA.

We confirmed a high prevalence of HCV-seropositivity among blood donors in Bangui and the subsequent high rate of HCV RNA viremic blood donations. To offset the major risk for transfusion-acquired HCV in Central Africa we recommend screening donated blood for anti-HCV. When laboratory facilities to perform ELISA are not available, the Quik Pack system,

a simple reliable method for detecting anti-HCV antibodies in human serum that requires neither complex reagent preparation nor expensive instrumentation, could prove useful.

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Immunization of Peacekeeping Forces[1]

To the Editor: The immunization status of military contingents arriving from different nations for peacekeeping missions may vary widely. This variation results from lack of information, coordination, and financial support.

For larger missions, the United Nations (UN) Headquarters issues recommendations about needed vaccines; recently, operations officers have consulted World Health Organization experts before issuing recommendations, and their advice, which takes into account epidemiologic data in the host country, has improved. Medical officers who develop recommendations for smaller missions must consider the pathogenic agent; environment; host efficacy, safety, and price of preventive measures; and legal and ethical aspects.

Data on the incidence of vaccine-preventable diseases within a military population that had similar duties in the same location are rarely available. When data from the respective region are not available, disease incidence or prevalence in the host country may be substituted. These data, however, may be misleading since the military often does not have the same lifestyle as the native population. Plague, for instance, had an incidence rate of 8 per 100,000 in Namibia, but not a single case was reported in the South African Armed Forces (unpub. SAMS report: Disease Profile of South West Africa, 1989). If epidemiologic documentation for a host country is not available, data from neighboring countries may be useful.

Traveler's diarrhea is the most frequent health problem abroad (1,2). Although the diarrhea is self-limited and lasts an average of 1 day with appropriate treatment (4 days without), the unproductive time may be detrimental to a military mission. Oral vaccines against the three most frequent causes of traveler's diarrhea (enterotoxigenic *Escherichia coli*, *Campylobacter* spp., and rotavirus [1,2]) are being developed; the latter will be available soon (3). Hepatitis A, most frequent among the vaccine-preventable diseases (4), is 10 to 100 times more frequent than typhoid fever (4,5). Hepatitis B occurs mainly in expatriates, but infections have also been observed in tourists who have had unprotected casual sex (6). The incidence rate of rabies is unknown, but animal bites that may result in rabies virus transmission and thus necessitate postexposure prophylaxis are frequent (7). Only anecdotal cases of diphtheria, tetanus, and tuberculosis have been reported (8). Poliomyelitis, yellow fever, Japanese encephalitis, and plague occur only in limited parts of the world (5). The situation may rapidly change as

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epidemics occur (e.g., diphtheria in eastern Europe in the early and mid-1990s) (9). If needed, the World Health Organization can provide information on confirmed and unconfirmed epidemics on a weekly basis.

Travel and peacekeeping mission statistics share similarities. In Namibia, the South African Armed Forces had most often observed hepatitis (unspecified), with rare cases of tuberculosis, typhoid, and meningitis (unpub. SAMS report: Disease Profile of South West Africa, 1989), as did the UN mission to Namibia, where within 12 months and with 7,114 employees, seven cases of hepatitis (mostly hepatitis A, some unspecified) occurred (10). No other vaccine-preventable infections were diagnosed in this UN mission.

Considering both risk (on the basis of incidence rates) and impact of infection, the priority for immunization (from highest to lowest) is as follows: hepatitis A, hepatitis B, rabies, poliomyelitis, yellow fever, typhoid fever, influenza, diphtheria, tetanus, meningococcal disease, Japanese encephalitis, cholera, and measles. To administer all vaccines would be extremely costly and may also result in an increased rate of adverse side-effects. Immunizations against the more frequent, more severe infections should be given priority.

If a mission is limited to one season, environmental factors of that respective season should be considered. This general rule is more important for vector-borne than for vaccine-preventable infections, except for influenza and meningococcal disease.

Persons who are already immune (because of previous immunization or immunity after infection) need not be vaccinated. The latter cause is particularly often true of hepatitis A; troops recruited in developing countries have an anti-hepatitis A virus seroprevalence rate close to 100% (11). Hepatitis B immunization, except for non- and low-responders, probably grants lifelong protection (12); the same is likely for measles vaccine.

Sometimes the host country may require proof of some specific vaccination based on the International Health Regulations (13), currently under fundamental revision to become a more effective tool in preventing the spread of infections that may be a global hazard (14).

In addition to adequate epidemiologic information and coordination between the military, international health organizations, and the host country, successful intervention efforts require thorough knowledge of vaccine characteristics with varying rates of efficacy and duration of protection. Cost-benefit evaluations, which would be very desirable, are unlikely in areas of political instability.

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Sexually Transmitted Diseases in Ukraine

To the Editor: With the political changes in eastern Europe in the last 10 years have come social and economic changes (1). Ukraine not

only faces almost insurmountable problems as it tries to form a new government, it also faces many serious health issues including sexually transmitted diseases (STDs).

Surveillance data from the Ukrainian STD Center from January 1, 1989, through December 31, 1995, were analyzed on the basis of reports received through 1997. In western Europe, the incidence of syphilis and gonorrhea declined from 1980 to 1991 to less than 2% per 100,000 persons for syphilis and less than 20% per 100,000 persons for gonorrhea. However, in Ukraine, since 1989, the notification rate of syphilis has skyrocketed—from 5 per 100,000 persons in 1990 to 170 in 1995. In some regions, this rate exceeds 220 cases per 100,000 persons. Moreover, cases among children younger than 14 years of age are also increasing. In 1995, the syphilis rate for persons older than 30 years of age was 170 per 100,000; 600 per 100,000 girls younger than 15 years of age; and 1,550 to 2,000 per 100,000 girls 15 to 16 years of age. The large number of girls with the disease is in part due to teenage prostitution (1).

Most syphilis and gonorrhea cases are attributed to sexual transmission. Explanations of this phenomenon include the rapid growth of the sex industry, increasing numbers of homeless persons and refugees in Ukrainian cities, poor diagnostic facilities, punitive legislation that reduces the likelihood of going to treatment services, and limited or inadequate treatment (2).

The Ukrainian government is reviewing its arrangements for the control of STDs, including HIV/AIDS, to identify clear objectives and priorities. Education and treatment would be effective in preventing the spread of STDs in Ukraine, but these measures are inadequately funded (3). Evaluation and risk reduction are also great weapons in preventing the spread of STDs (4). However, the response of the local and world communities has been inadequate in stemming a major STD epidemic in Ukraine.

United Nation's Children's Fund (UNICEF) is developing a long-term program in Ukraine with a focus on STDs in adolescents and youth. This comprehensive program will tackle not only STDs but other related issues, such as HIV and teenagers' reproductive health (5).

Greater coordination of the agencies responsible for STD control in Ukraine will be sought, together with an expansion of health promotion and prevention projects for young persons and groups at high risk (6). An effective strategy for the control of STDs in Ukraine will, therefore, need to find ways to modify current programs and the way they interact to create effective control interventions.

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Yellow Fever Vaccine

To the Editor: Monath et al. (1) outlined existing facilities for distribution of yellow fever vaccines in the United States and pointed to difficulties for prospective vaccinees in remote locations. Their recommendation that primary health-care providers be allowed to dispense yellow fever vaccination merits serious consideration. Acceptance of such a strategy in the United States would inevitably be emulated elsewhere. Nevertheless, before such a strategy is approved, vaccine potency should be monitored at distribution points, and a sample of vaccine recipients should be examined for vaccine-induced immune response.

In Nigeria, systematic investigation of yellow fever vaccine distribution and transportation to remote locations has found loss in vaccine potency. Vaccine in storage sites and immuniza-

tion centers in Lagos was fully potent, but potency in Osun and Oyo was 016 $\log_{10}$ to 0.22 $\log_{10}$ lower than the stipulated level (2). Furthermore, the titer of two vaccine lots that had been frozen after reconstitution from their lyophilized state dropped from the initial 3.15 $\log_{10}$ to 3.53 $\log_{10}$ to zero.

If the United States were to implement an extended strategy, similar studies of vaccine lots should be conducted to determine whether every vaccinee has received a full dose of yellow fever vaccine. In Illinois during the early 1970s, weak links in maintenance of refrigeration facilities and use of outdated vaccines in vials exposed to the sun for long hours were reported for live poliovirus vaccines (3). In the Northern Territory of Australia, examination of 144 vials of hepatitis B vaccine formulations during transport to immunization centers showed that 47.5% had been exposed to temperatures of -3°C or lower (4).

Assays of the potency of yellow fever vaccine, as well as quantification of vaccine-induced neutralizing antibody, is a multistep procedure that relies on inoculation of mice or Vero or polysaccharide cells (5). The successful “take” of yellow fever vaccine can be determined starting the second postvaccination day by demonstrable viremia detected by reverse-transcriptase polymerase chain reaction and by marked increases in neopterin, beta2-microglobulin, and circulating $CD8^+$ cells (6). Alternatively, elevated levels of tumor necrosis factor and interleukin-1 receptor antagonists on day two after vaccination (7) could be used to monitor the success of vaccinations by primary-care providers in remote areas in the United States (1) and elsewhere.

During the 1990s, isolation of yellow fever virus was reported in persons with a nonspecific febrile illness that did not meet the case definition of yellow fever (8). Air travel by such persons to the United States, which has areas infested by *Aedes aegypti,* could initiate yellow fever epidemics; because these travelers would have a nonspecific febrile illness, they would escape the existing surveillance network.

In conclusion, introducing yellow fever immunizations by primary health-care providers would be ideal, only with a concurrent plan to monitor vaccine potency at immunization centers and obtain in vitro evidence of a successful vaccine take. Such a strategy would blunt yellow fever–associated deaths, illnesses, and symptomless viral carriage in the community.

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Yellow Fever Vaccine—Reply to S. Arya

To the Editor: Dr. Arya correctly points out that there have been problems with degradation of live viral vaccines, including yellow fever vaccines, that have not been properly handled and stored at the point of use. However, in the United States and western Europe, yellow fever vaccines are stabilized and require the same storage facilities at the point of use as other vaccines routinely distributed by family physicians and pediatricians. Varicella vaccine (and even measles vaccine) is less stable than yellow fever vaccine but is distributed to all registered physicians in the United States. Since vaccines and other perishable medicines are typically

shipped by overnight courier services using qualified methods that ensure maintenance of low temperature, there is no barrier to use of a similar system for yellow fever vaccine.

Empirical testing for antibody, viremia, or even surrogate markers of T-cell activation may be useful; however, it is difficult and expensive, involves unvalidated tests with unknown sensitivity and specificity, and is unnecessary, except under very special circumstances. A more direct measure of vaccine stability is direct potency measurement of samples stored at the point of use, as was done in the cited study in Nigeria by Adu et al. However, given the current controls on vaccine distribution in the United States, we do not believe that there would be a need to validate vaccine effectiveness at point of use in the event of a change of policy with respect to vaccinating centers. The cold-chain infrastructure and the training of medical personnel in vaccine storage and administration may not provide the same assurances in other countries. While our suggested changes to the system of yellow fever distribution may improve vaccine coverage and have other desirable benefits in the United States, they would not be appropriate for less stable systems for vaccine supply and use.

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54th International Northwestern Conference on Diseases in Nature Communicable to Man August 15-18, 1999

The 54th International Northwestern Conference on Diseases in Nature Communicable to Man at Utah State University, Logan, UT, is scheduled for August 15-18, 1999. Call for abstracts in the following general categories are proposed, but may be modified: viral zoonoses, bacterial zoonoses, water and foodborne infections, vector-borne diseases, and curiosa et exotica. The deadline for abstracts is April 15, 1999.

The R.R. Parker Memorial Address will be presented by Jesse Goodman, Senior Advisor for Medical Programs, U.S. Food and Drug Administration. To obtain program announcements and registration forms, make hotel reservations, or submit abstracts, contact one of the following persons: Dhitinut Ratnapradipa, scientific program coordinator, e-mail: dratnapr@health.utah.edu; or Robert Elbel, meeting coordinator, e-mail: elbel@bioscience.utah.edu, tel:(801) 581-4816, fax: (801) 581-4668. Program information is also available at the following web site: http://www.mosquito.org./upcoming.html.

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EMERGING INFECTIOUS DISEASES Tracking trends and analyzing new and reemerging infectious disease issues around the world

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-639-4856 (tel), 404-639-3075 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Spanish and French translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Int Med 1997:126[1]36-47) (http://www.acponline.org/journals/resource/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/tsd/serials/lji.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in either (TIFF), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title in full. List the first six authors followed by "et al."

Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send files, slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS C-12, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.